

MULTIPLE MOLECULAR FORMS OF ENZYMES

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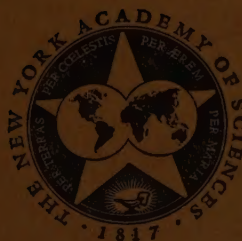
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INTRODUCTORY REMARKS

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It has long been recognized that enzymes from different species that have the same substrate specificity and catalyze the same reaction may nevertheless differ markedly in various other properties. It had been commonly inferred, but rarely demonstrated, that such isodynamic enzymes from diverse tissues of an organism also differed. The practice of identifying an enzyme by the species and tissue or organ from which it originated has, accordingly, been widely and prudently followed. Evidence that this designation is not necessarily sufficient to specify an enzyme appeared over two decades ago when naturally occurring multiple forms of pepsin and artificially produced multiple forms of chymotrypsin were reported. Occasional similar reports for other enzymes were published in the following 15 years, but it was not until 1957 that a considerable number of papers began to appear on this subject. Since then interest in multiple molecular forms of enzymes, as judged by number of publications, has increased rapidly. Far more publications on enzyme heterogeneity have appeared during the last two or three years than the total of such publications previously.

It is apparent that enzyme heterogeneity is a common phenomenon. More than 30 enzymes have been shown to exist in multiple forms within individual organisms. They have been observed in both plants and animals, in unicellular microorganisms as well as multicellular species. They have been distinguished on the basis of a variety of characteristics including electrophoretic and chromatographic behavior, serological specificity, differential solubility, and differential response with coenzyme analogues. Heterogeneity has been observed in enzymes with widely divergent functions, the oxidases as well as the anaerobic dehydrogenases, the dehydrases as well as the hydrolases, to mention but a few.

The importance of the study of multiple forms of enzymes stems in part from their frequent but by no means universal occurrence. Their study promises to expand our knowledge in a variety of fields ranging from embryology and the study of evolution to physiology and pathology. Study of them has already proved that it has clinical diagnostic applications. The omnipresent possibility of heterogeneity in an enzyme isolated from any unstudied source is clearly an important factor to be considered in any enzymatic investigation.

The very existence of closely related multiple forms of enzymes poses many questions. Does the presence of more than one form of an enzyme offer any advantage to an organism? Are the multiple forms synthesized individually or are they all derived from one fundamental form? How do the multiple forms differ chemically and structurally from each other? What is the relationship of multiple forms of enzymes to phylogeny and ontogeny? Papers included in this monograph contain data that enable us to begin answering some of these questions.

Some of the papers included here concern multiple forms of enzymes that show some variation in substrate specificities and perhaps should be considered distinctly different enzymes. Likewise, at least one paper deals with multiple forms that may exist in the laboratory but not in nature. Indeed, some of the other multiple forms reported might, unknowingly, belong in one or the other of these two categories. A consideration of these peripheral cases is important if only because it is of assistance in delineating and defining naturally occurring multiple molecular forms of enzymes in a strict sense.

The widespread occurrence of multiple molecular forms of enzymes presents novel problems of nomenclature. Should a group of closely related proteins occurring in one organism and having identical substrate specificity and catalytic function be considered separate enzymes or variants of one enzyme? What should such a group of enzymes be called and how should each individual form be designated? What criteria should be used to identify such a group of enzymes? How may one be sure that the multiple forms of an enzyme which one observes exist naturally and do not arise from changes occurring during their isolation? These are questions that various papers in this monograph answer in different ways. In an attempt to achieve some uniformity in nomenclature these questions were discussed by a panel and in open discussion on the last day of the conference on which this publication is based.

CHROMATOGRAPHIC HETEROGENEITY OF SOME ENZYMES IN NORMAL TISSUES AND TUMORS*

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Chromatography on ion-exchange cellulose derivatives, as developed by Peterson and Sober, is a method that has been used to purify many enzymes from various sources.¹ In our laboratory, soluble proteins of liver, brain, and other organs have been fractionated by modifications of this method, the validity and resolution of which have been demonstrated by the localization of enzyme activities as sharp peaks in the chromatograms^{2,3} (FIGURE 1).

One problem that generally arises when continuous methods of protein fractionation (such as zone electrophoresis or chromatography) are applied to crude extracts of organs is that of the appearance of multiple, separable forms of the same activity. This phenomenon is shown for hexokinase, glucose-6-phosphate dehydrogenase, and acid phosphatase from rat liver in FIGURE 1. In the chromatograms multiple forms of malic dehydrogenase, glutamic-oxalacetic transaminase, and β -glucuronidase activities from liver have been found as well.²

The proportions of each form of a multiple form enzyme are characteristic of the organ of origin, as shown by Markert and Moller⁴ and others, and as shown (FIGURE 2) in chromatograms of rat organ extracts.³

Multiple forms could be chemically variant proteins with identical or closely similar enzyme activities, presumably with a gene determining each molecular form, or determining at least one peptide chain in the forms; or they could be the same protein molecule existing in different forms, arising for example by binding to other molecules or by aggregation; or they could be artifacts of preparation, such as denatured or degraded forms of the same protein molecule.

One of the criteria used to show that multiple forms of an enzyme activity represent in fact different molecules is that each form, when separately put through the fractionation procedure again, should not be converted into the other forms. This is evidenced, during the refractionation, by the quantitative recovery of activity as a single form. This criterion has been met for glucose-6-phosphate dehydrogenase and glutamic-oxalacetic transaminase from rat liver,² and for the multiple forms of acid phosphatase (see under *Results*, below).

However, this criterion is not sufficient, and there must be evidence independent of the fractionation procedure, since conceivably the conversion of one form to another, if it can occur, might not occur appreciably under the conditions of the fractionation. For example, binding of a molecule to the enzyme might be tight or irreversible, or the enzyme might be degraded or denatured irreversibly, retaining its activity but behaving differently chromatographically. Such independent evidence might include kinetic, immunological, physical, or chemical differences demonstrated among the forms. In this paper are described the separation of multiple forms of acid phosphatase activity from

* The work described in this article was supported in part by Research Grant C 5228 Met. from the National Cancer Institute, Public Health Service, Bethesda, Md.

liver and brain, and the demonstration that the forms differ in their enzymatic properties.

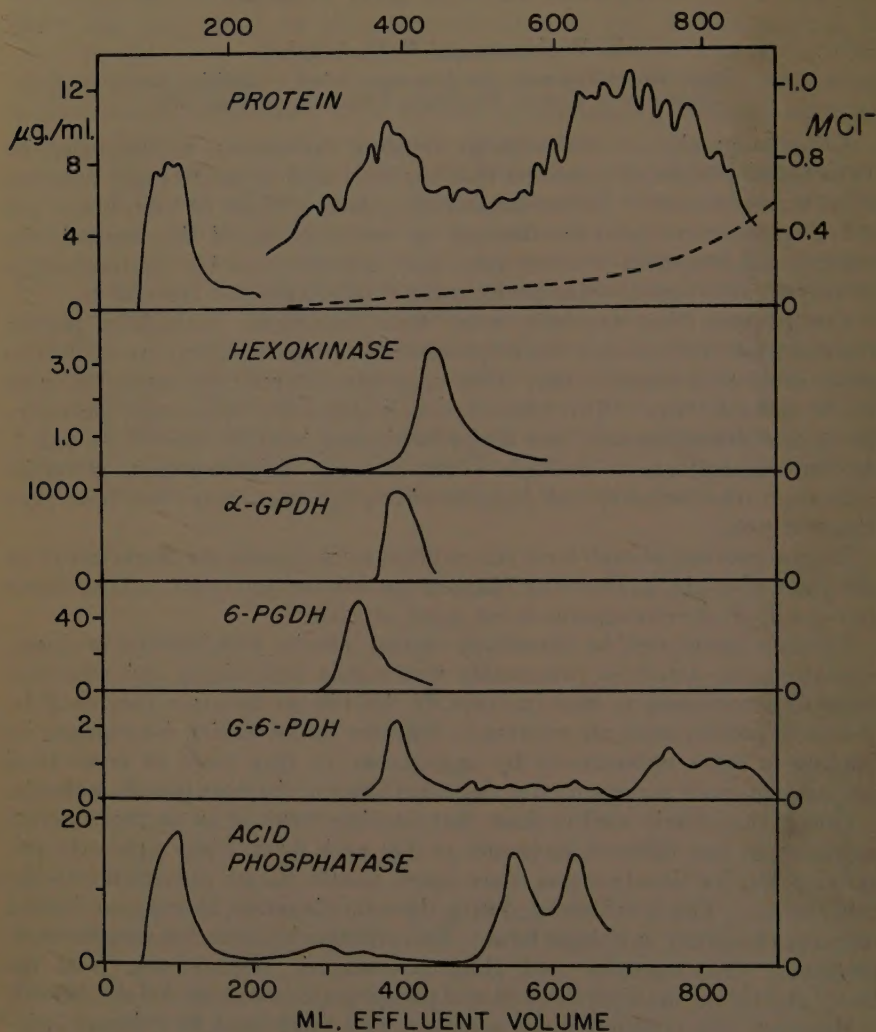


FIGURE 1. Separation of rat-liver soluble proteins on DEAE-cellulose. Enzyme activities are in mM/hour.

Methods

Organs or tumors removed from decapitated and bled animals were homogenized in 3 volumes of 0.01 *M* tris-phosphate pH 8.0. The rat liver and brain extracts, and the rabbit liver extracts were centrifuged at 100,000 *g* for 60 min., the other extracts at 15,000 *g* for 120 min. The supernatants were equilibrated with the same buffer by dialysis or by passing them through Sephadex columns, and then they were eluted by a chloride gradient developed with a 9-chambered

gradient device.* The gradient had the shape shown in FIGURES 1 and 3; the limit buffer was 1 *M* NaCl, 50 mM sodium phosphate, pH 6.2. All buffers were prepared in 10 mM 2-mercaptoethanol. Protein was measured by the Lowry⁶ method. Acid phosphatase activity was assayed (unless otherwise

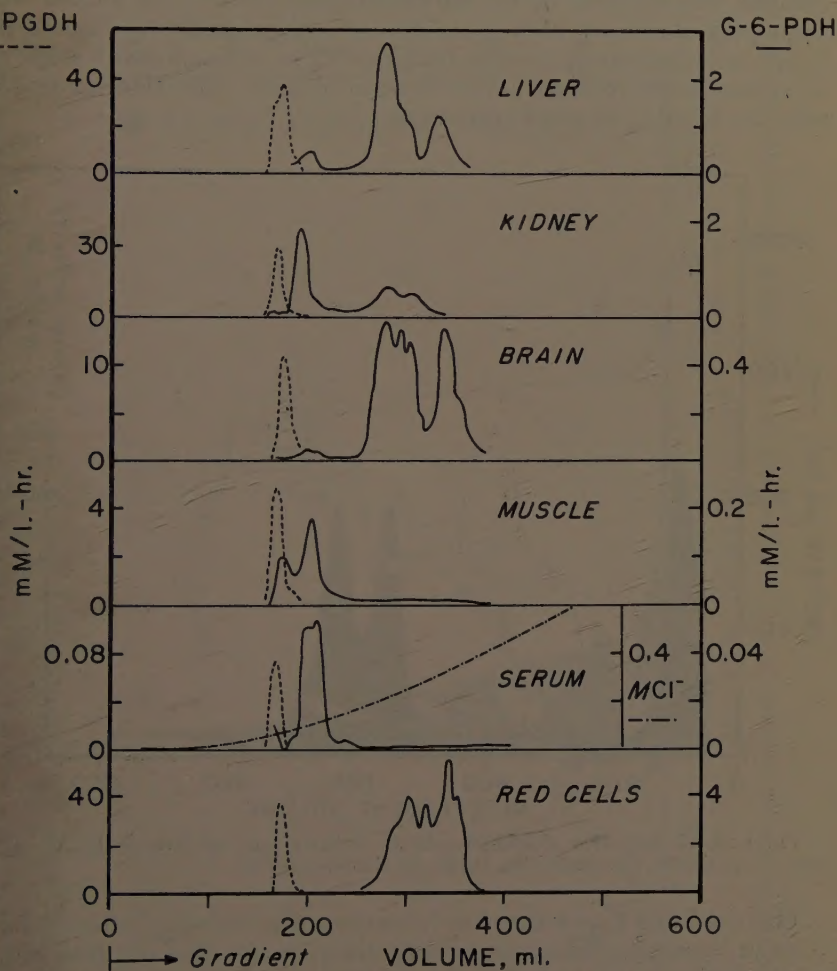


FIGURE 2. Localization of glucose-6-phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconic dehydrogenase (6-PGDH) in chromatograms of soluble proteins from different rat tissues. Enzyme activities are in mM/hour.

indicated) with 20 mM *p*-nitrophenyl phosphate as substrate in 0.1 *M* sodium citrate pH 5.2 in the presence of 0.05 per cent bovine plasma albumin.

Results

Acid phosphatase of rat liver was separated chromatographically (FIGURE 3) into 3 major and 1 minor peaks of activity. Recovery of activity was 80 to 90

*Technicon "Autograd."⁵

per cent. Peaks II and III were not separated from each other by a parabolic chloride gradient, and were split apart only when the gradient was expanded in the region of 0.1 *M* chloride, as shown in FIGURE 3. Peak I did not bind to DEAE-cellulose. Peak I, when rechromatographed on CM-(carboxymethyl)-cellulose, was adsorbed by the ion-exchanger at *pH* 6.0, but yielded only a single peak of activity when eluted by a phosphate gradient. Each of the 4 forms was rechromatographed on DEAE-cellulose, and each gave a single peak with quantitative recovery at the expected position. The three major forms were also found in rat brain (FIGURE 4).

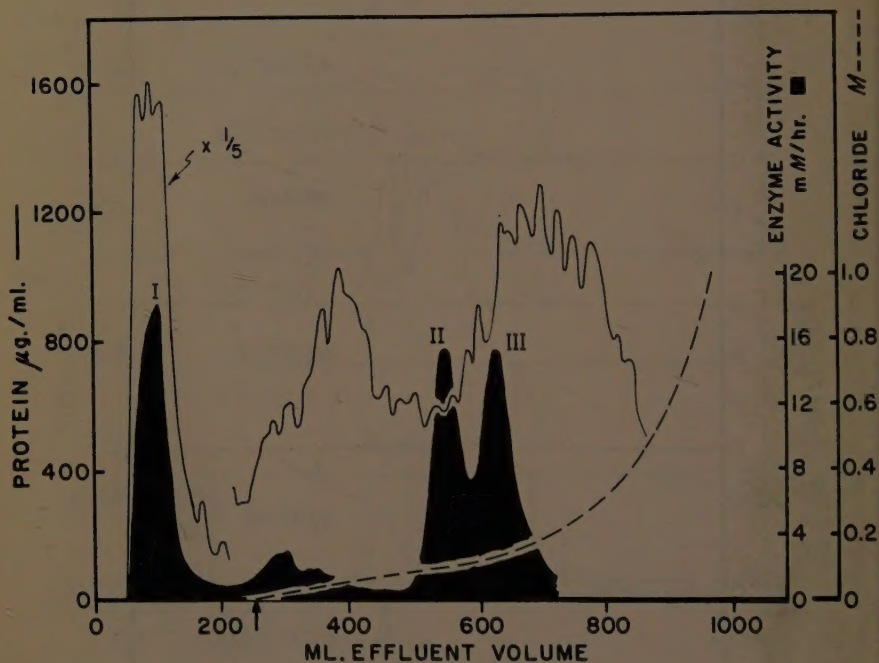


FIGURE 3. Localization of acid-phosphatase activity from rat liver in DEAE-cellulose column fractions. The dashed line shows the chloride gradient.

FIGURES 5 and 6 show the plots for determining Michaelis constants for the 3 major forms from liver. Essentially similar results were obtained with the corresponding forms of brain. The 3 forms for liver differ in *pH* optima (FIGURE 7) and in sensitivity to fluoride inhibition (FIGURE 8). TABLE 1 summarizes the enzymatic properties of the chromatographically separated forms of acid phosphatase from rat, rabbit, and chicken organs. In addition to the differences in *pH* optima, Michaelis constants, and fluoride inhibition, there are variations among the forms in their relative specificities for the substrates α - and β -glycerophosphate and *p*-nitrophenyl phosphate.

Chicken liver (TABLE 1) yielded two forms of acid phosphatase. In contrast to those from rat liver, the basic peak (peak I not adsorbed to DEAE-cellulose) was the form that had the higher *pH* optimum, the lower *K_m*, and that was

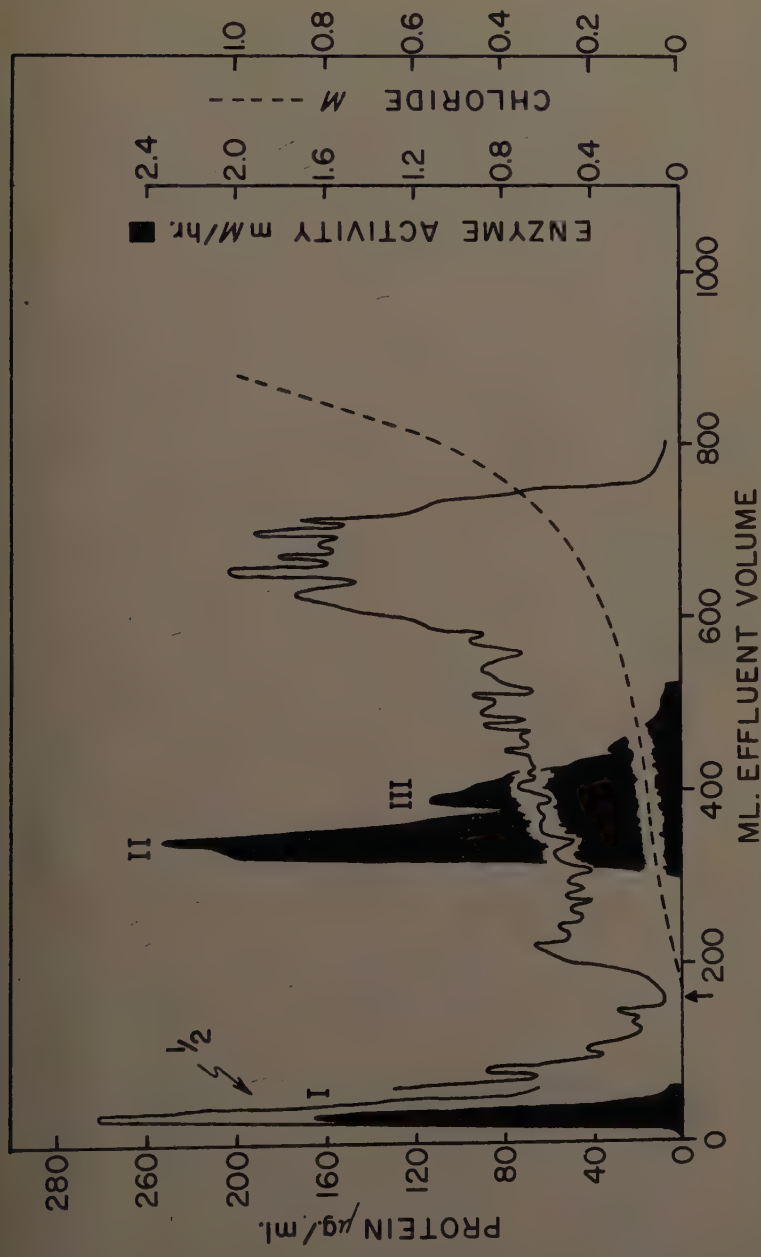


FIGURE 4. Localization of acid-phosphatase activity from rat brain in DEAE-cellulose column fractions. The dashed line shows the chloride gradient.

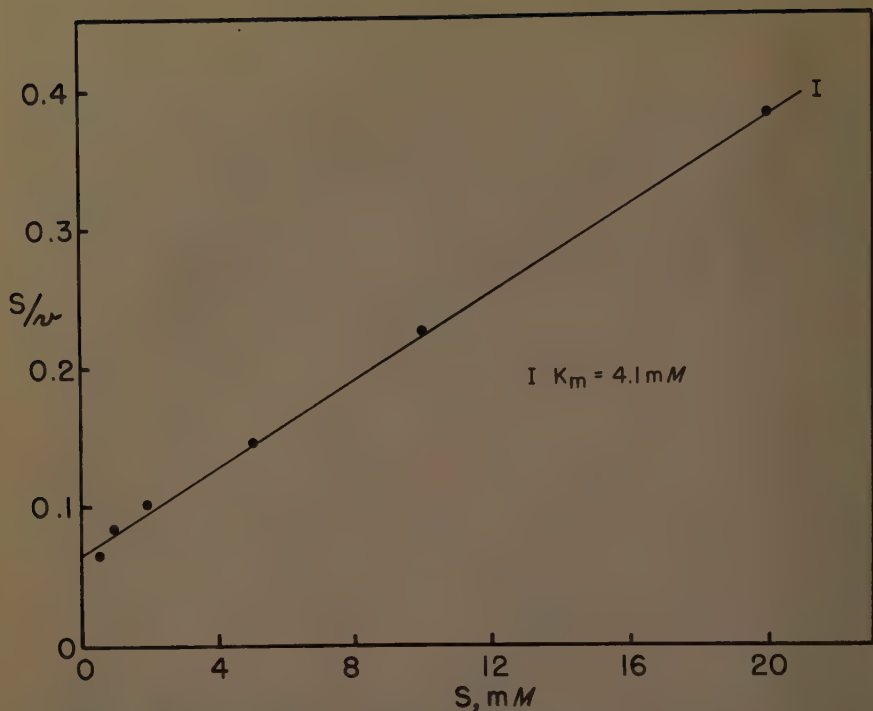


FIGURE 5. Michaelis constants for rat-liver acid phosphatase forms with *p*-nitrophenyl phosphate as substrate in pH 5.2 citrate buffer at 37° C.

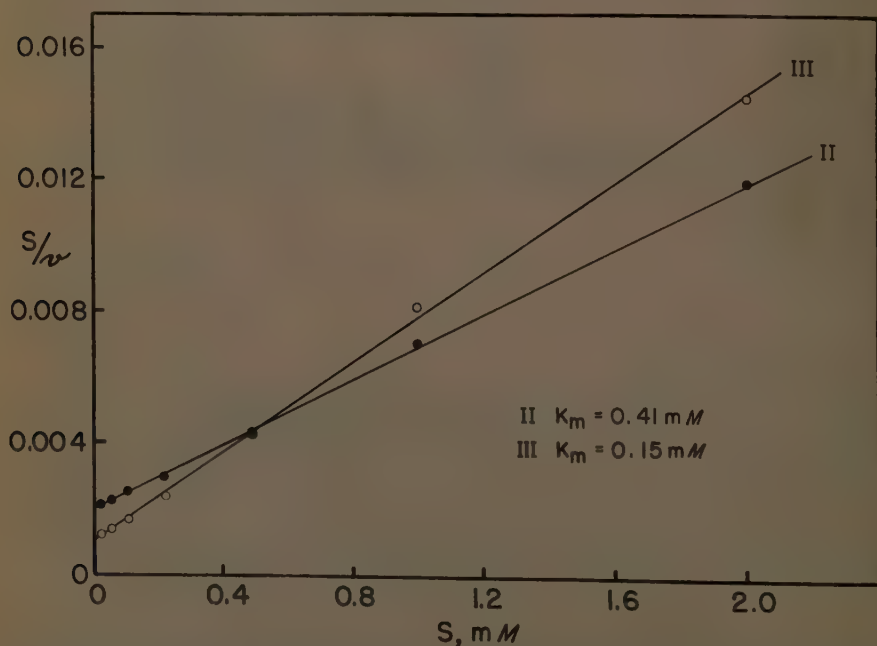


FIGURE 6. Michaelis constants for rat-liver acid phosphatase forms with *p*-nitrophenyl phosphate as substrate in pH 5.2 citrate buffer at 37° C.

less sensitive to fluoride inhibition. Similar results were obtained for chicken brain. Each of the two forms was rechromatographed and each gave a single peak at the expected position.

Rabbit liver (TABLE 1) gave, in addition to 3 forms comparable to the major forms of rat liver, a fourth form that was not eluted from the column until 180 mM chloride was reached, and was inhibited by fluoride.

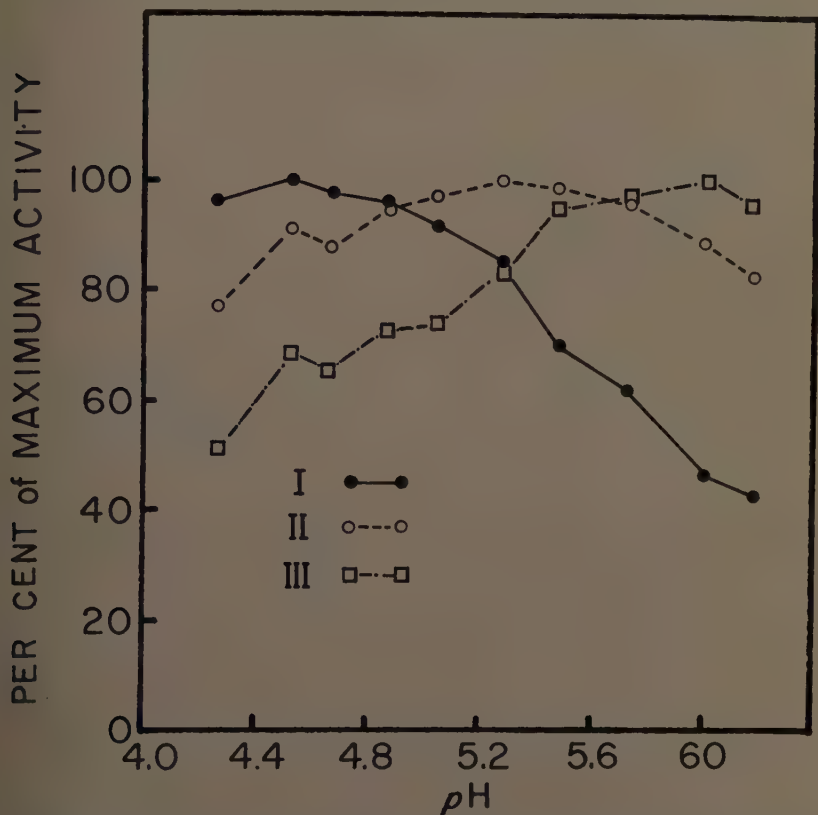


FIGURE 7. Optimum pH values for rat-liver acid phosphatase forms with *p*-nitrophenyl phosphate as substrate in citrate buffers.

Some rat and mouse tumors (TABLE 2), when compared to the normal organs of origin, have relatively a greater proportion of the basic, fluoride sensitive peak I of the enzyme, but the chromatographic and enzymatic properties of the tumor enzymes were indistinguishable from the normal ones.

Discussion

Multiple forms of acid phosphatase exist in extracts from organs of several species: rat, mouse, rabbit, and chicken. In general, forms with indistinguishable chromatographic and enzymatic properties were found in liver and brain of the same species. For example, the three major forms from rat liver were

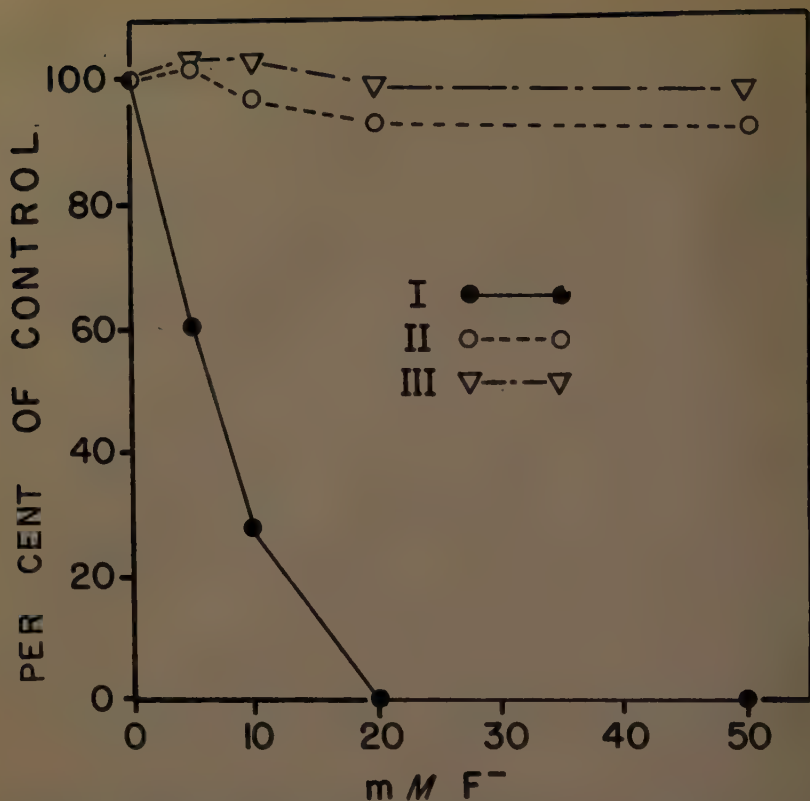


FIGURE 8. Effect of fluoride on rat-liver acid phosphatase forms with *p*-nitrophenyl phosphate as substrate in pH 5.2 citrate.

TABLE 1
FORMS OF ACID PHOSPHATASE

	mM Cl ⁻ eluted	pH opt.	% F ⁻ inhibition	α-GP†/β-GP	PNPP‡/β-GP	Km PNPP (mM)
Rat liver						
I	0	4.0	100	0.3	5	4.1
Ia	20	4.9	80	0.5	4	3.7
II	100	5.2	2	4.5	41	0.4
III	135	6.0	6	9.3	40	0.1
Rat brain						
I	0	4.0	87	—	—	4.6
II	105	4.8	8	—	—	0.4
III	190	5.8	0	—	—	0.1
Rabbit liver						
I	0	4.0	64	—	—	2.8
II	70	5.2	0	—	—	0.5
III	100	5.6	5	—	—	0.5
IV	180	4.7	55	—	—	—
Chicken liver						
I	0	5.6	20	—	—	0.5
II	90	4.5	100	—	—	1.5

* 20 mM NaF.

† Ratio of activities with α- and β-glycerophosphates (50 mM).

‡ Ratio of activities with *p*-nitrophenyl phosphate (20 mM) and β-glycerophosphate (50 mM).

similar to those of rat brain. Also, the forms seen in tumors resembled those from normal tissues of the same species, but their relative proportions differed. Similar results have been obtained for other tumor enzymes.⁷⁻⁹

Peaks I and Ia (TABLE 1) of rat liver were similar to each other in chromatographic and enzymatic properties, as were peaks II and III, suggesting that those pairs are structurally similar proteins.

In general, forms with different chromatographic and enzymatic properties were found in different species. There was no correlation between chromatographic and enzymatic properties of the acid phosphatase forms in different species (TABLE 1).

TABLE 2
ACID PHOSPHATASE IN TUMORS

	Per cent of total activity	
	Peak I	Peak II*
Normal rat liver	20	80
Hepatoma	75	25
Normal mouse epithelium	15	85
Papilloma	10	90
Squamous cell carcinoma	60	40

* Sum of Peaks II and III (see FIGURE 3).

The results suggest that the multiple forms of acid phosphatase found in animal organs are distinct molecular forms since they differ not only in chromatographic properties (reflecting protein charge and size) but also in enzymatic properties. Furthermore, it is likely that there is a genetic basis for the multiple forms of the enzyme, since they appear to be species-specific but not organ-specific.

References

1. TURBA, F. 1960. *Advances in Enzymology*. **22**: 417.
2. MOORE, B. W. & R. H. LEE. 1960. *J. Biol. Chem.* **235**: 1359.
3. MOORE, B. W. 1959. *Federation Proc.* **18**: 283.
4. MARKERT, C. L. & F. MOLLER. 1959. *Proc. Natl. Acad. Sci. (U.S.)*. **45**: 753.
5. PETERSON, E. A. & H. A. SOBER. 1959. *Anal. Chem.* **31**: 857.
6. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL. 1951. *J. Biol. Chem.* **193**: 265.
7. ANGELETTI, P. U., B. W. MOORE, S. SOLARIC & V. SUNTZEFF. 1960. *Proc. Soc. Exptl. Biol. Med.* **103**: 329.
8. ANGELETTI, P. U., V. SUNTZEFF & B. W. MOORE. 1960. *Cancer Research*. **20**: 1229.
9. ANGELETTI, P. U., B. W. MOORE & V. SUNTZEFF. 1960. *Ibid.* **20**: 1592.

MOLECULAR VARIATION IN SIMILAR ENZYMES FROM DIFFERENT SPECIES*

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Although it has been known for many years that similar enzymes isolated from different organisms may demonstrate wide variations in their physical properties,¹⁻¹⁰ this fact has excited little interest, perhaps because of the wide divergences of the sources. Rather more interest has been elicited by the now well-established observation that different proteins with similar enzyme activities may exist within the same organism and even within the same tissue.¹¹⁻²⁰ It seemed possible that some of the variations of certain enzymes within a species might be simply a limited manifestation of a wide variation in molecular structure among different species. These experiments were therefore intended to demonstrate whether variations among species are exceptional or common. It was found that, in fact, they are the rule.

Starch gel electrophoresis was used to separate the different proteins, which were then characterized by their enzymatic activities and relative mobilities.

The enzymes studied were esterases, phosphatases, catalases, and peroxidases. They were generally selected for their ease of detection but esterase activity was specially studied because many enzymes have secondary esterase activity²¹⁻²³ and therefore they might be considered to represent a spectrum of similar kinds of enzymes.

*Materials and Methods*²⁴

Electrophoresis. The apparatus and general methods were essentially the same as those described by Smithies.²⁵ Poulik's²⁶ discontinuous system of buffers was employed, and the samples were introduced into the gels in small pieces of Whatman 3MM filter paper inserted into slits made with a razor blade.

Detection of enzymes. After completion of the run the gel was removed and split horizontally in the orthodox manner. One half of the gel was usually stained for protein with Amido black 10B and the other half was stained for enzyme activity. In order to compare relative mobilities different specimens were inserted side by side in the same gels and run together. Esterases were determined by a slight modification of Gomori's²⁷ modification of Nachlas and Seligman's²⁸ method, in which naphthol released from α -naphthyl acetate is allowed to couple with tetrazotized *o*-dianisidine to form an insoluble dye at the site of the enzyme. Phosphatases were detected by a similar technique,⁹ except that α -naphthyl phosphate was substituted for α -naphthyl acetate. Peroxidases were located by the benzidine method.⁹ For the determination of catalase activity a technique first suggested by A. J. Hale was employed. The gels were submerged in a dilute solution of hydrogen peroxide (1 per cent 100 vol.), washed with water and then immersed in a 4 per cent solution of potas-

* Supported by grants from the Scottish Hospital Endowments Research Trust.

† Beit Memorial Fellow.

sium iodide acidified with acetic acid. Iodine released from the iodide by peroxide stained the starch gel a deep blue except in those areas where the peroxide was destroyed by catalase activity.

Preparation of extracts. Extracts of tissues were usually prepared by grinding them with 0.15 *M* sodium chloride or balanced salt solution in a Potter homogenizer, subjecting the suspension to ultrasonic treatment (50 w, 20 kc./sec.) for 15 to 20 min. and then centrifuging at 35,000 g for 2 hours at 0° C. to remove insoluble material. Preliminary homogenization was not found to be necessary when cell suspensions were used, and for this material 2 to 3 min. ultrasonic treatment was adequate.

Results

The effect of the method of preparation on the type of pattern obtained was first investigated. Samples of mouse liver were extracted with water, 0.15 *M* NaCl, Hanks' balanced salt solution,²⁹ Tris buffers at pH 7.3, and pH 8.3, and phosphate buffers at pH 7.3 and pH 8.3. Samples of each were applied to gels



FIGURE 1. Photograph of an esterase zymogram of three mouse kidney preparations, including one from which two bands were absent.

immediately after extraction and after varying periods of ultrasonic treatment. They were then stained for esterases. The same patterns were obtained when the tissues were extracted with water, NaCl solution or balanced salt solution, and the patterns were not altered by prolonged ultrasonic treatment although the yield increased. All the buffer solutions, however, produced preparations that gave slightly different patterns, and these patterns underwent some alteration during ultrasonic treatment. These observations indicated that a few of the bands might be artefacts, possibly polymers of the same protein unit. However, the majority of the bands appeared in the same positions on the gels irrespective of the methods of preparation.

When a standard method of preparation was employed, highly reproducible patterns were always obtained from the same organ within a species. For instance from 46 mice we prepared extracts of liver and kidney and obtained identical zymograms for each tissue in 44 animals. The two exceptions were of considerable interest themselves since they provided evidence to suggest that certain enzymes at least are different proteins which may be genetically absent in some animals. One of these is illustrated in FIGURE 1. In this particular gel three mouse kidney preparations were run simultaneously. It is clear that in almost all respects the three gels exhibit the same patterns. Preparation G, however, lacks two bands present in the others. It is interesting that one of

these two bands was also absent from the liver preparation from the same animal, whereas the other was present.

When preparations from different species were compared with those from the mouse, patterns characteristic of each species were found. FIGURES 2 and 3 show the comparative patterns for livers and sera respectively. It is clear that the majority of the bands are unique for each species, and where similar mobilities are observed it is not possible to exclude coincidence. Apparently

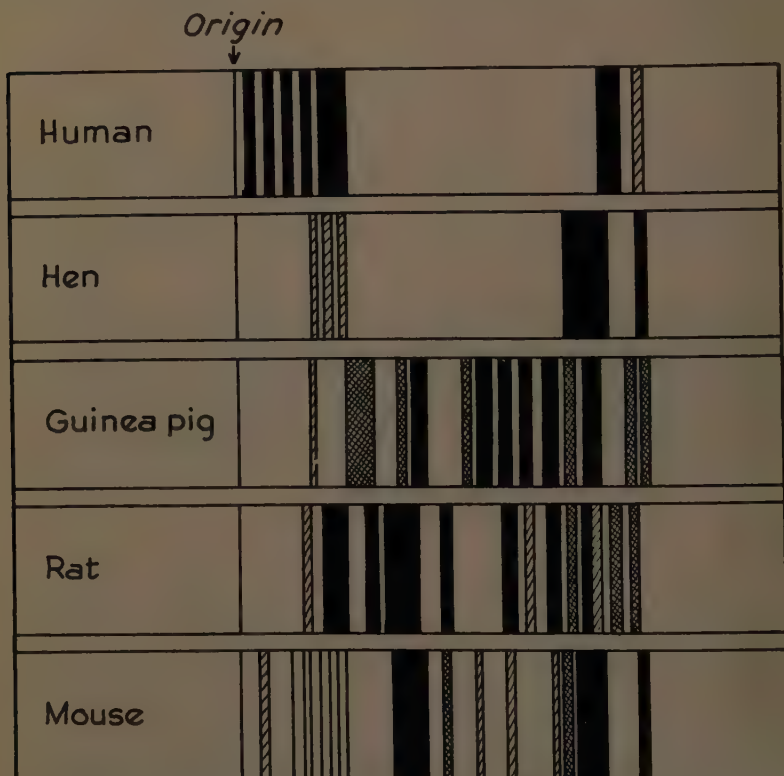


FIGURE 2. Esterase zymograms of liver preparations from different species.

the individual enzymes differ from species to species. The numbers of proteins with enzyme activity vary also.

Still another interesting difference can be observed in comparing the variation in tissue patterns in different species. In an animal such as the mouse there is a considerable variation in the number of esterase bands seen in zymograms from different tissues (Markert and Hunter¹⁷). We found no less than 16 bands in liver preparations and smaller numbers in other tissues. However, within this species the patterns observed in different tissues represented different combinations of the same limited group of enzymes, all of which could be identified in the liver. Some other animals, for example the guinea-pig and

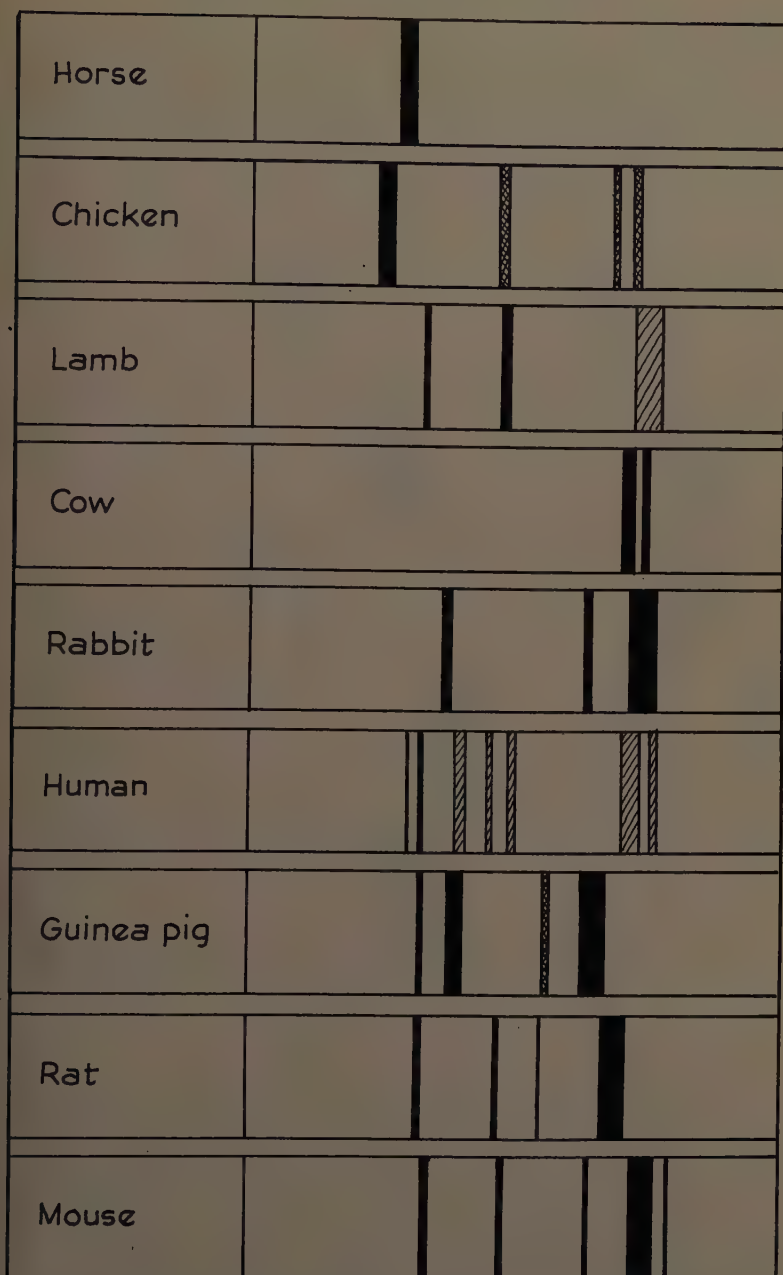


FIGURE 3. Esterase zymograms of serum from different species.

rat, display the same kind of variation but, on the other hand others, notably man, show remarkably little variation from one organ to another.

While the esterases themselves probably represent a heterogeneous group of loosely related enzymes it was obviously desirable to investigate the behavior of enzymes with other substrate specificities as well. Some of the results of

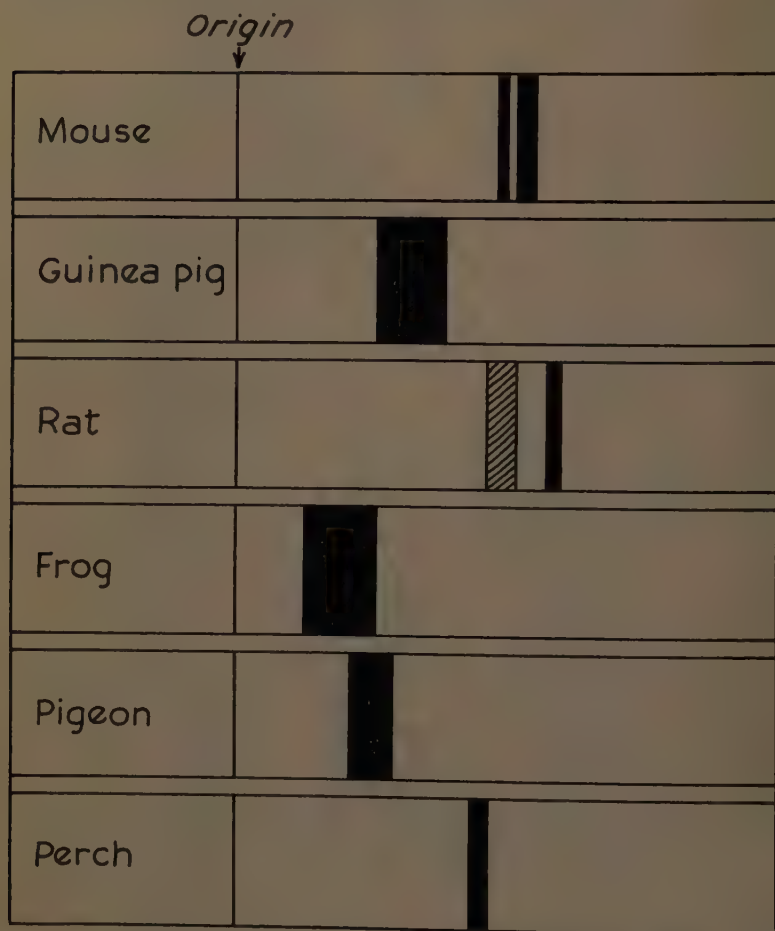


FIGURE 4. Alkaline phosphatase zymograms of liver preparations from different species.

these studies are summarized in FIGURES 4 to 6. Most of these enzymes are represented by only one or two bands on starch gel electrophoresis, but again it is clear that the electrophoretic mobilities of enzymes from different and even fairly closely related species are rarely the same.

The question arises whether some of the differences among organs and species might be the result of environmental rather than genetic factors. We tested this possibility by investigating the patterns to be found in cultured cell strains from different species maintained in essentially the same environment for several

years. As FIGURE 7 shows, all human cell strains exhibited a typical human esterase pattern as distinct from mouse cells. This strongly suggests that most of these enzymes are constitutive in nature and genetically stable. A further deliberate attempt was made to induce changes in the esterases of these cells by culturing them in the presence of a high concentration of an aromatic ester (acetylsalicylic acid). No alteration in the zymogram pattern was obtained.

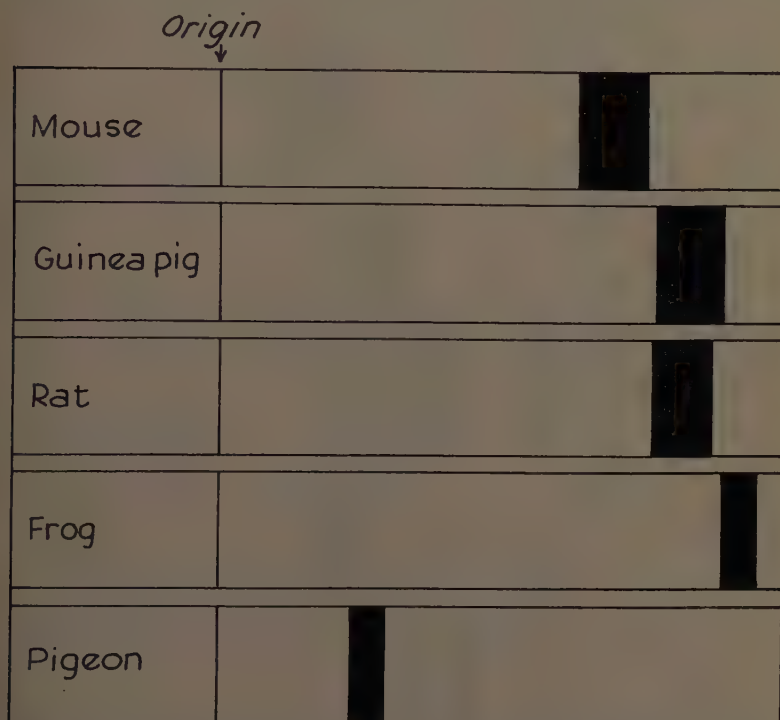


FIGURE 5. Peroxidase zymograms of liver preparations from different species.

Discussion

The results of these and similar studies performed by other investigators¹⁻¹⁰ suggest that molecular variation among similar enzymes from different species, far from being an exceptional phenomenon, may be the rule, and that consequently enormous numbers of proteins with similar enzyme activities may exist. The generalization may extend to proteins and polypeptides other than enzymes. For instance species differences have been demonstrated in adrenocortrophin,³⁰ haemoglobin,³¹ insulin,³² melanocyte-stimulating hormone,³³ and vasopressin,³⁴ among others. In some of these, notably insulin,³² the species differences have been shown to involve only a few amino acids and sometimes only one. An analogous situation has been demonstrated within the human species by Hunt and Ingram³⁵ in certain haemoglobins that differ from each other in a single amino acid, the differences being genetically determined.

These conclusions contrast strikingly with the observed conservation of metabolic pathways at all levels of evolutionary development.³⁶ The answer to the paradox probably lies in the existence of large areas of enzyme molecules that can vary greatly without resulting in a serious loss of activity,³⁷⁻⁴² whereas other areas may be less flexible. There are grounds for believing that the

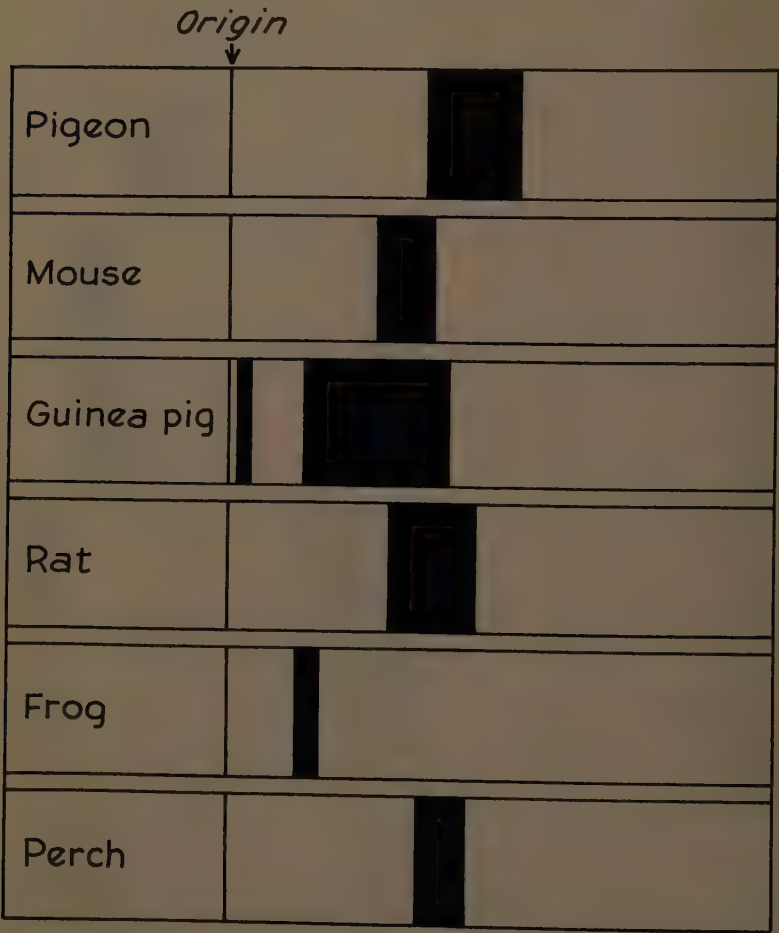


FIGURE 6. Catalase zymograms of liver preparations from different species.

active centers of enzyme and protein hormone molecules have somewhat rigorous structural limitations,⁴³⁻⁴⁷ yet findings such as those of Kaplan⁴⁸ and Jolles⁴⁹ suggest that even within these areas some variation may be compatible with the same enzyme function. Consequently it is easy to appreciate the fact that while many genetic mistakes may result in the exclusion of the resulting proteins by selective pressure many others may lead to variants that have full enzymatic activity and can persist. An enormous number of func-

tionally similar proteins may therefore occur throughout the biosphere, and certain examples of isozymes may be but a local manifestation of this.

Having proposed this explanation we can perhaps make out a good case for it on other grounds. For one thing the wide range of immunological differences observed among living things indicates that there are enormous numbers of different proteins in nature: far more than would seem to be required for life processes if each kind of protein were represented by a very few similar molecules. Finally, if all the proteins with the same activities had essentially the same

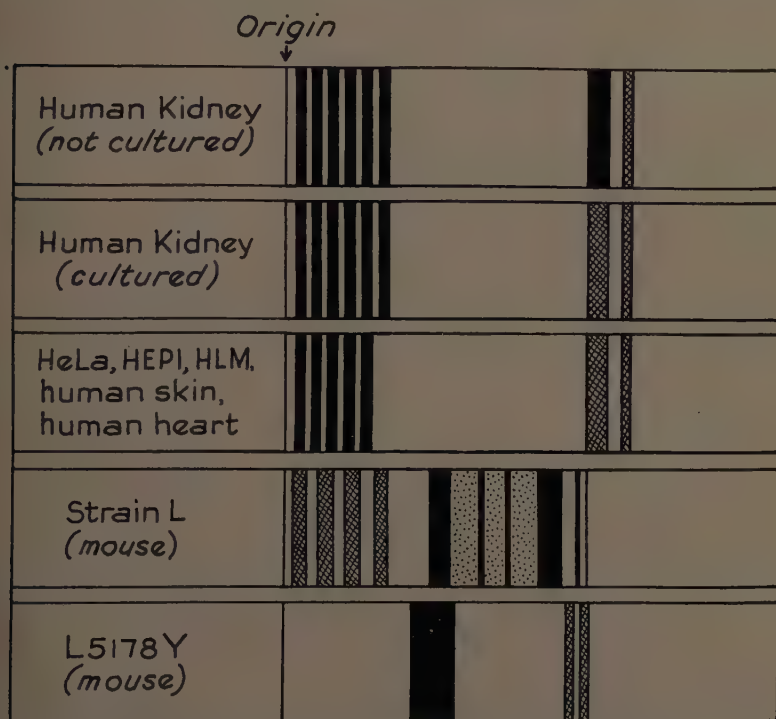


FIGURE 7. Esterase zymograms from cultured cell strains compared with human kidney. HeLa, HEPI, HLM are all of human origin.

structure it would not be easy for new functional molecules to arise by accidents which involved the alteration of only one or two amino acids. With an enormous pool of different protein forms, however, a trap is laid for the rare fortunate accident, and opens the way to a smooth pattern of evolution.

Summary

The electrophoretic behavior of esterases, phosphatases, peroxidases, and catalases obtained from a variety of animal species was studied. Almost invariably differences between species were demonstrated, and this is taken to indicate that structural variations in similar proteins from one species to another

may be the rule. It is suggested that many examples of isozymes may be local manifestations of this, and the significance of this conclusion in relation to molecular evolution is discussed.

References

1. AQVIST, S. E. G. & C. B. ANFENSEN. 1959. *J. Biol. Chem.* **234**: 1112-1117.
2. ANFENSEN, C. B., S. E. G. AQVIST, J. P. COOKE & B. J. JONSSON. 1959. *J. Biol. Chem.* **234**: 1118-1123.
3. HENION, W. F. & E. W. SUTHERLAND. 1957. *J. Biol. Chem.* **224**: 477-488.
4. TAYLOR, J. F., A. A. GREEN & G. T. CORI. 1948. *J. Biol. Chem.* **173**: 591-604.
5. HAUPT, F. & H. GIERSEBERG. 1958. *Naturwissenschaften*. **45**: 268-269.
6. PLAGEMANN, P. G. W., K. F. GREGORY & F. WRÓBLEWSKI. 1960. *J. Biol. Chem.* **235**: 2282-2293.
7. KAPLAN, N. O., M. M. CIOTTI & F. E. STOLZENBACH. 1956. *J. Biol. Chem.* **221**: 833-844.
8. AUGUSTINSSON, K. B. 1958. *Nature*. **181**: 1786-1789.
9. MARKERT, C. L. & F. MØLLER. 1959. *Proc. Natl. Acad. Sci.* **45**(5): 753-763.
10. CINADER, B. 1957. *Ann. Rev. Microbiol.* **11**: 371-390.
11. RYLE, A. P. & R. R. PORTER. 1959. *Biochem. J.* **73**: 75-86.
12. TANG, J. 1959. *J. Biol. Chem.* **234**: 1174-1178.
13. MITIDIERI, E. L., L. P. RIBERIO, O. R. AFFONSO & G. G. VILLELA. 1955. *Biochem. et Biophys. Acta*. **17**: 587.
14. MILLS, G. T., J. PAUL & E. B. SMITH. 1953. *Biochem. J.* **53**: 232-245.
15. MEISTER, A. 1950. *J. Biol. Chem.* **184**: 117-129.
16. WIELAND, T. & G. PFLEIDERER. 1957. *Biochem. Z.* **329**: 112-116.
17. MARKERT, C. L. & R. L. HUNTER. 1959. *J. Histochem. and Cytochem.* **7**: 42-49.
18. JOLLES, G. & C. FROGMAGEOT. 1954. *Biochem. et Biophys. Acta*. **14**: 219-230.
19. TIMASHEFF, S. N., J. M. STURTEVANT & M. BIER. 1956. *Arch. Biochem. Biophys.* **63**: 243-246.
20. VESELL, E. S. & A. G. BEARN. 1958. *Ann. N. Y. Acad. Sci.* **75**(1): 286-291.
21. TINOCO, I., JR. 1958. *Arch. Biochem. Biophys.* **76**: 148-160.
22. KAUFMANN, S., G. W. SCHWERT & H. NEURATH. 1948. *Arch. Biochem.* **17**: 203-205.
23. SHIPPEY, S. S. & F. BINKLEY. 1958. *J. Biol. Chem.* **230**: 699-705.
24. PAUL, J. & P. FOTTELL. 1961. *Biochem. J.* **78**: 418-424.
25. SMITHIES, O. 1955. *Biochem. J.* **61**: 629-641.
26. POULIK, M. D. 1957. *Nature*. **180**: 1477-1479.
27. GOMORI, G. 1952. *Microscopic Histochemistry*. Univ. Chicago Press. Chicago, Ill.
28. NACHLAS, M. M. & A. M. SELIGMAN. 1949. *J. Natl. Cancer Inst.* **9**: 415-425.
29. HANKS, J. H. & R. E. WALLACE. 1949. *Proc. Soc. Exptl. Biol. Med.* **71**: 196-200.
30. WHITE, W. F. & A. M. GROSS. 1957. *J. Am. Chem. Soc.* **79**: 1141-1145.
31. OZAWA, H. & F. SATAKE. 1955. *Biochem. J. (Japan)*. **42**: 641-648.
32. HARRIS, J. I., F. SANGER & M. A. NAUGHTON. 1956. *Arch. Biochem. Biophys.* **65**: 427-438.
33. ACHER, R. 1960. *Ann. Rev. Biochem.* **29**: 547-576.
34. POPENOE, E. A., H. C. LAWLER & V. DU VIGNEAUD. 1952. *J. Am. Chem. Soc.* **74**: 3713.
35. HUNT, J. A. & V. M. INGRAM. 1958. *Nature*. **181**: 1062-1063.
36. BALDWIN, E. 1948. *Introduction to Comparative Biochemistry*. Cambridge Univ. Press. Cambridge, England.
37. RICHARDS, F. M. & P. J. VETHAYATHIL. 1959. *J. Biol. Chem.* **234**: 1459-1465.
38. RESNICK, F. M., J. R. CARTER & G. KALNITSKY. 1959. *J. Biol. Chem.* **234**: 1711-1713.
39. ROGERS, W. I. & G. KALNITSKY. 1957. *Biochem. et Biophys. Acta*. **23**: 525-532.
40. HILL, R. L. & E. L. SMITH. 1958. *J. Biol. Chem.* **231**: 117-134.
41. EVANS, R. L. & H. A. SAROFF. 1957. *J. Biol. Chem.* **228**: 295-304.
42. SMITH, E. L., R. L. HILL & A. BORMAN. 1958. *Biochem. et Biophys. Acta*. **29**: 207-208.
43. OOSTERBANN, R. A., P. KUNST, J. VAN ROTTERDAM & J. A. COHEN. 1958. *Biochem. et Biophys. Acta*. **27**: 549-555.
44. PORTER, G. R., H. N. RYDON & J. A. SCHOFIELD. 1958. *Nature*. **182**: 927.
45. SMITH, E. L. & M. J. PARKER. 1958. *J. Biol. Chem.* **233**: 1387-1391.
46. SMITH, E. L. 1958. *J. Biol. Chem.* **233**: 1392-1397.
47. NEURATH, H. 1957. *Advances in Protein Chem.* **12**: 320-386.
48. KAPLAN, N. O., M. M. CIOTTI, M. HAMOLSKY & R. E. BIEBER. 1960. *Science*. **131**: 392-397.
49. JOLLES, P. 1960. *Lysozyme*. In *The Enzymes*. **4**: P. D. Boyer, H. A. Lardy & K. Myrback, Eds. Academic Press. New York, N. Y.

Discussion of the Paper

A. SAMUELS (*Department of Pathology, Dartmouth Medical School, Hanover, N. H.*): With respect to multiple molecular forms of enzymes, it is mentioned that the kinetic characteristics of similar enzymes from the same organs of different species are similar. Is there any compilation of such evidence in the literature? I have found that 5'-adenylic acid deaminase from skeletal muscle of chicken and rabbit has identical K_m values, as reported by Ya-Pin Lee* and myself.†

* JOURNAL OF BIOLOGICAL CHEMISTRY. 1957. **227**: 999.

† THE PHYSIOLOGIST. 1957. **1**: 24.

PHYSICOCHEMICAL NATURE OF ISOZYMES*

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The properties of living cells are in large part a reflection of the enzymes that they contain, and these enzymes are in turn a manifestation of the activity of the nucleic acids of the cells. We have all become familiar with the prevailing hypothesis that relates the primary structure of enzymes (the linear sequence of amino acids) to a corresponding sequence of nucleotides in DNA and/or RNA. This relationship was long ago aptly summed up as the one gene-one-enzyme hypothesis. One reasonable prediction of this hypothesis is that a homozygous organism should synthesize identical molecular replicas of each of its various protein molecules, and this should be true for all the cells of the organism. We were surprised, therefore, to discover from our own work and by a perusal of the literature that single enzymes commonly existed in multiple molecular forms, or *isozymes*, within the tissues or cells of a single organism. These isozymes are not artifacts of analysis but exhibit characteristic patterns of distribution in each tissue (FIGURE 1). Moreover the tissue patterns are species specific (FIGURE 2). The characteristically different isozyme patterns of adult tissues must have arisen during the course of embryonic development, and direct analysis shows this to be true. The adult pattern is the end product of a long sequence of gradual changes during ontogeny. The remarkable specificity in isozyme pattern that characterizes each tissue implies a significant physiological role for isozymes even though they are essentially alike in enzymatic activity. The existence of isozymes poses important biological problems as well as problems in the physical chemistry of proteins. It is of great importance to know whether the distinctions among isozymes lie in the primary, secondary, or tertiary structure of the molecule, for a knowledge of such differences may provide an insight into the biological significance of multiple molecular forms of enzymes and, perhaps, also into the mechanisms of protein synthesis. Accordingly we present the results of a physicochemical study on the recrystallized, separated isozymes of lactate dehydrogenase obtained from beef hearts.

Materials and Methods

In this study crystalline lactate dehydrogenase (LDH) was prepared from beef hearts according to the method of Straub (1940). The following compounds were employed: DPN, DPNH, TPN, and the 3-acetylpyridine, pyridine 3-aldehyde, deamino, and thionicotinamide analogs of DPN.† Sodium pyruvate, sodium lactate, sodium p-chloro-mercuribenzoate,‡ and potassium oxamate were also used.§

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† From the Pabst Brewing Company, Milwaukee, Wis.

‡ Purchased from Sigma Chemical Company, St. Louis, Mo.

§ From Matheson Coleman and Bell, Norwood, Ohio.

Moving boundary electrophoretic analyses were carried out in a Perkin-Elmer model 38 apparatus. Quantitative separation of isozymes in crystalline preparations was achieved by use of a Munktell cellulose column, essentially as suggested by Flodin and Kupke (1956). The basic procedures previously outlined by Markert and Møller (1959) for the electrophoretic resolution of

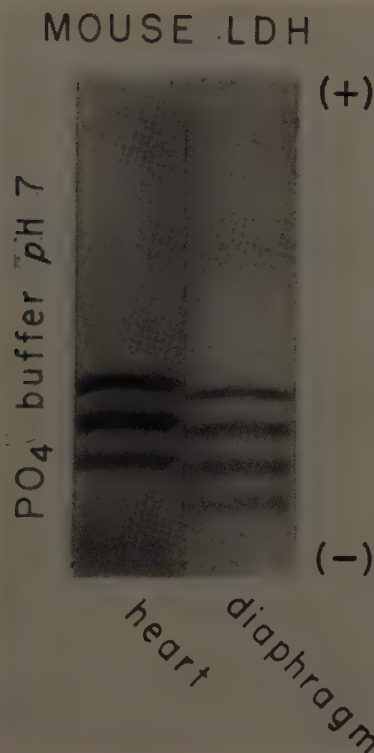


FIGURE 1. Starch gel electrophoresis zymograms stained to reveal the isozymes of LDH from heart and diaphragm of the mouse. These two organs have similar though distinct isozymic patterns. To reveal the location of LDH activity after electrophoresis the starch strips are incubated anaerobically at 37° C. in the dark in the following mixture: 0.5 *M* hydrazine, 1.0 ml.; 0.5 *M* sodium lactate, 6.0 ml.; DPN at 10 mg./ml., 2.0 ml.; phenazine methosulfate at 0.2 mg./ml., 2.0 ml.; neotetrazolium chloride (sat. sol.), 4.1 ml.; 0.2 *M* Tris buffer pH 8.3, 15.0 ml.

tissue homogenates and crystalline preparations of LDH in starch gels were followed except for one important innovation: phenazine methosulfate (Nachlas *et al.*, 1960) replaced diaphorase in the reaction mixture used to visualize the location of LDH on the starch gels.

For ultracentrifugal analysis a Spinco model E ultracentrifuge was employed; experiments were conducted at a rotor speed of 59,780 r.p.m. and at temperatures maintained by means of the rotor temperature-control unit between 4° and 10° C. The sedimentation coefficients, reported as $S_{20,w}$, were calculated

from least square slopes as discussed by Schachman (1957) and corrected for the viscosity and density of water at 20° C. Diffusion constants, determined in a synthetic boundary cell (Pickels *et al.*, 1952), were calculated with the use of the first moment method and corrected for the centrifugal field and the time at which the boundary was established.

The partial specific volumes of the proteins were determined at 20° C. in a 2 ml. pycnometer calibrated with distilled water at that temperature. Ultra-

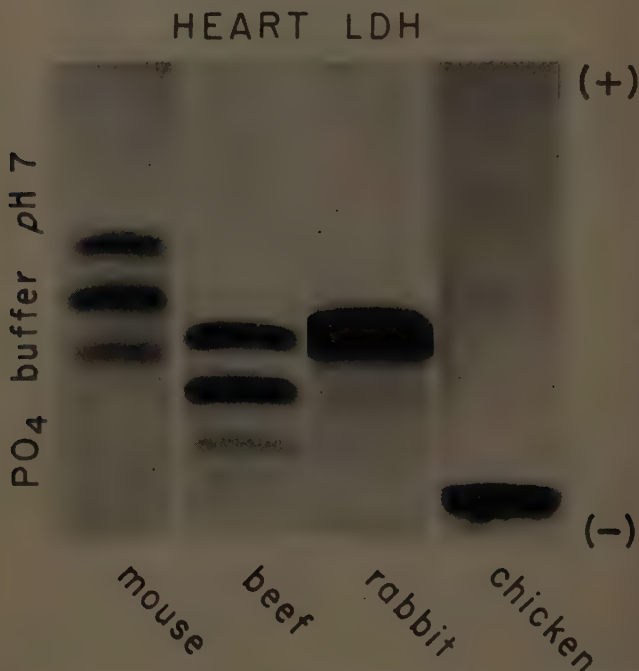


FIGURE 2. Zymograms showing LDH isozyme patterns in heart tissues of four different animals. Each pattern is species specific.

violet light absorption was measured with a Beckman model DU spectrophotometer equipped with a 1-cm. quartz cell. For ultraviolet measurements a 0.05 to 0.10 per cent solution of the enzyme was used. Optical rotation was measured with a Rudolph High Precision spectropolarimeter equipped with a Xenon lamp; the temperature was maintained at $15^{\circ} \pm 0.1^{\circ}$ C. by circulating water at that temperature through the jacket of a 10-cm. cell.

The activity of the enzyme was assayed spectrophotometrically at a wave length of 340 $m\mu$ with the use of a Beckman DU spectrophotometer. For assays of enzyme activity with analogs of DPN, wave lengths were chosen at the absorption peaks for each analog (Anderson *et al.*, 1959). The reactants were added to a 3-ml. cuvette in the following order: 0.3 ml. of 0.1 *M* Tris

buffer, 0.15 ml. of 0.015 *M* coenzyme, 0.3 ml. of 0.2 *M* substrate, and distilled water to a volume of 3 ml. For the pyruvate to lactate reaction a *pH* of 7.4 was used and, for the oxidation of lactate to pyruvate, a *pH* of 9.0 was used. The enzyme was made up in 0.1 *M* phosphate buffer at *pH* 7 and introduced at zero time. The mixture was stirred and the optical density was determined at 15-sec. intervals. The linear portion of the curve was used in calculating enzyme activity, one unit of which is defined as a change in optical density of 0.100/min. under the conditions of the standard assay system. Specific activity is defined as units of enzyme activity per milligram of protein. Protein concentration was determined by nesslerization after Kjeldahl digestion (Calvin, 1958).

Results

Physicochemical data. In the preparation of LDH, analysis of the initial crude homogenate by starch gel electrophoresis revealed the existence of three separable fractions with LDH activity. During the course of the purification procedure the isozymic pattern remained remarkably constant, and the crystalline preparation likewise contained 3 isozymes in relative concentrations similar to those found in the homogenate. Analysis of the crystalline preparation in the Tiselius apparatus over a *pH* range of 5 to 9 revealed the 2 major components. The quantity of the third component was at the limit of resolution in moving boundary electrophoresis, although quite evident in zone electrophoresis. The larger, faster migrating component (A) represents about 85 per cent of the total protein, the second (B) about 15 per cent, and the third less than 1 per cent. Typical patterns in moving boundary and starch gel electrophoresis are shown in FIGURE 3.

At *pH* 4.8 and ionic strength 0.1, the LDH begins to precipitate, thus precluding an extension of the electrophoretic analysis into more acid ranges. From the *pH*-mobility curves in acetate, phosphate, and veronal buffers with an ionic strength of 0.1, the isoelectric point was calculated by extrapolation to be 4.5 for A and 4.8 for B. These results are in agreement with the earlier work of Neilands (1952).

In order to separate quantitatively the first two isozymes we have used Porath's technique of zone electrophoresis in a column of Munktell cellulose powder. A typical elution diagram is shown in FIGURE 4. The recovery of protein with this method was about 90 per cent. The specific activity of the two separated isozymes as compared to the initial crystalline preparation did not vary by more than 5 per cent. When the separated fractions were collected, precipitated with ammonium sulfate, dialyzed, and electrophoresed in starch gel, each retained its specific mobility as shown in FIGURE 5. All separated fractions were homogeneous.

Analysis in the ultracentrifuge of the unfractionated enzyme and of each of the two isolated isozymes revealed only one single homogeneous symmetrical peak for each preparation. The values of the sedimentation constants extrapolated to zero protein concentration were calculated as 7.0 (A) and 7.1 (B). These values are not significantly different and are in agreement with the value reported for the unfractionated crystalline enzyme (Neilands, 1955).

The diffusion constants, although calculated with some uncertainty because of the method of determination, were estimated to be 5.10 for A and 5.15 for B. These values did not appear to vary with protein concentration and do not serve to distinguish the isozymes. The partial specific volume was found to be 0.750 in a single determination for each isozyme. From these measurements a calculation of the molecular weight by means of the Svedberg equation gave



FIGURE 3. Ascending (*left*) and descending (*right*) electrophoretic patterns in the Tiselius; phosphate buffer at pH 7 and ionic strength 0.07. The two components are isozymes of crystalline beef heart LDH. Below is a corresponding pattern in starch gel electrophoresis. The origin is at the left, and resolution was achieved in 12 hours at 4° C. with a voltage gradient of 6 v/cm. at pH 7.2.

133,650 for A and 134,260 for B; these values are in good agreement with the previously reported molecular weight of 135,000 for the unfractionated enzyme (Neilands, 1952). The frictional ratio calculated from the sedimentation and diffusion constants was 1.2 for both A and B. The axial ratios obtained from the frictional ratios by the use of Perrin's relation on the assumption that the LDH protein is an unhydrated ellipsoid were 4.5 for a prolate ellipsoid and 5.0 for an oblate ellipsoid (Svedberg and Pedersen, 1940). The various physical constants that have been measured or calculated in this investigation for these two isozymes of LDH are collected in TABLE 1.

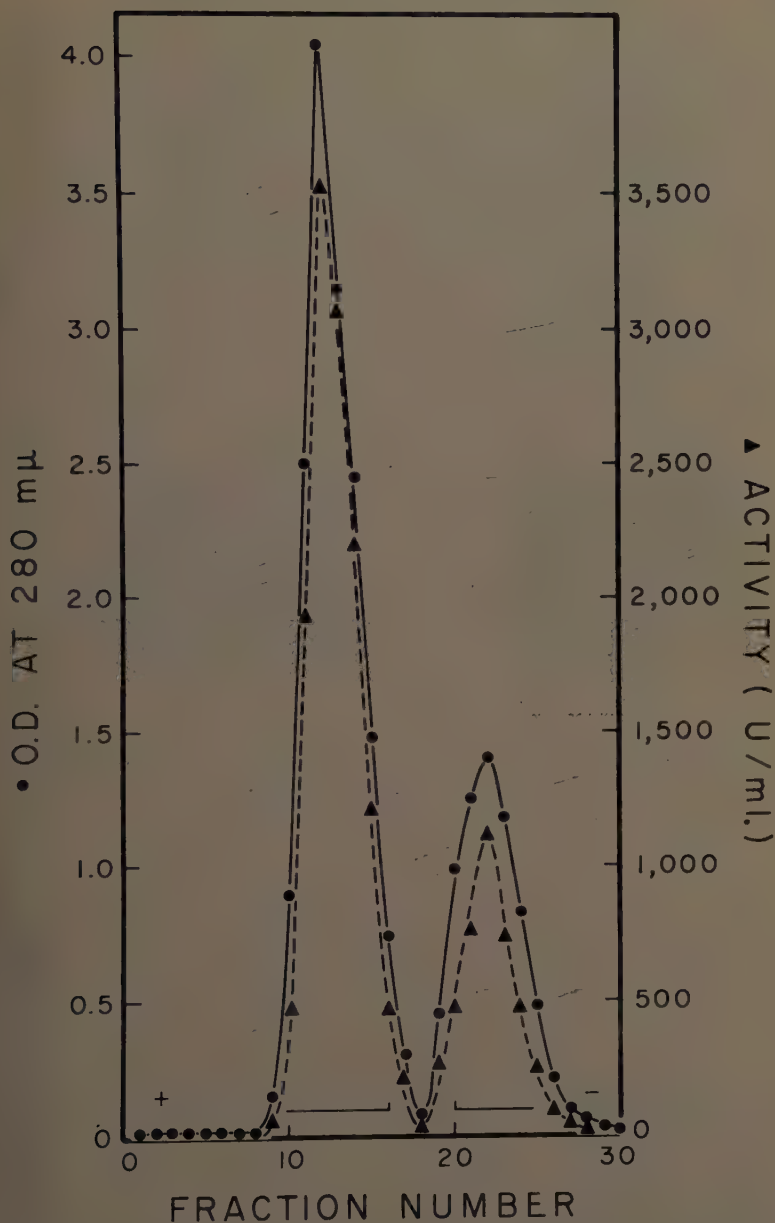


FIGURE 4. Typical elution pattern after electrophoresis in a column of cellulose powder; phosphate buffer at pH 7.2 and 0.03 ionic strength was used during electrophoresis and also as the eluting agent. The specific activity of the starting material was 564 units/mg. After fractionation the larger A isozyme showed specific activity of 590 units/mg. and the B isozyme 574 units/mg.

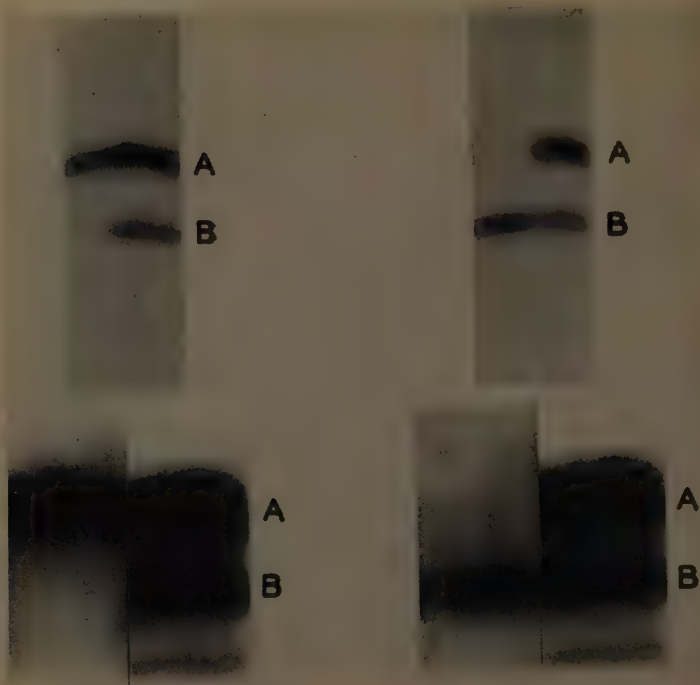


FIGURE 5. These zymograms demonstrate the purity and unchanged electrophoretic behavior of the isozymes separated on the cellulose column. The upper two starch strips are stained for protein; the lower two for LDH activity. The unfractionated crystalline enzyme was inserted as a control on the right half of each starch strip. On the left half was placed one of the separated isozymes. The origin and cathode are at the bottom and the anode at the top.

TABLE 1
PHYSICOCHEMICAL PROPERTIES OF ISOZYMES A AND B FROM CRYSTALLINE BEEF HEART LDH*

	A	B
Sedimentation coefficient ($S_{20,w}^0 \times 10^{-13}$ cm./sec.)	7.0	7.1
Diffusion coefficient ($D_{20,w} \times 10^{-7}$ cm. ² /sec.)	5.10	5.15
Partial specific volume (W_{20} ml./g.)	0.750	0.750
Molecular weight	134,000	134,000
Frictional ratio (f/f_0)	1.2	1.2
Isoelectric point	4.5	4.8
Electrophoretic mobility (phosphate buffer ionic strength 0.07, pH 7.02 $\times 10^{-6}$ cm. ² /volt second)	4.9	4.0
Molar extinction coefficient ($\epsilon \times 10^3$ mole/liter)	2.10	1.96

* These data reveal a significant difference between isozymes A and B in electrophoretic mobility and in isoelectric point. In all other characteristics the data do not so far demonstrate any difference.

In order to examine the stability of the protein, the unfractionated enzyme was subjected to ultracentrifugation at different pH values and ionic strengths. As shown in TABLE 2, the sedimentation constant is independent of pH within the range of 5.8 to 11. At a pH of 12, however, the LDH becomes polydisperse in the ultracentrifuge and shows 2 peaks with sedimentation constants of 4.05 and 6.65. Ultracentrifugation of the enzyme in solutions of different ionic strengths did not alter the sedimentation constant except at the very high ionic strength of 1.10, where a decrease of 18 per cent was observed, but this is probably only a charge effect in the medium and does not indicate any change in the enzyme molecule.

Measurements designed to reveal some aspects of the chemical composition of isozymes have not thus far uncovered any significant differences between them. The molar extinction coefficient at 280 $m\mu$, the peak of absorption, was 2.10 for A and 1.96 for B, with a range of variability in the determinations of not more than 8 per cent.

TABLE 2
SEDIMENTATION CONSTANTS OF BEEF LACTATE DEHYDROGENASE

Ionic strength	Buffer	pH	$S_{20,w}$
0.03	phosphate	7.06	7.10
0.05	phosphate	7.06	6.96
0.10	phosphate	7.06	7.10
0.50	phosphate	7.10	6.16
1.10	phosphate	7.00	5.77
0.10	phosphate	5.80	6.90
0.10	TRIS	8.20	6.88
0.10	TRIS	8.95	6.85
0.10	glycine	10	6.70
0.10	glycine	11	6.75

The tryptophan + tyrosine content of the LDH was calculated from the absorption spectrum in 0.1 N NaOH. At this high pH (12.7) the phenolic groups of the tyrosyl residues are ionized, thus exposing 2 maxima in the ultraviolet absorption spectrum, one for tryptophan at 284 $m\mu$, the other for tyrosine at 290 $m\mu$. The slope of the line drawn tangent to these two maxima indicates the ratio of tyrosine to tryptophan. From the extinction coefficients and the maximal absorbance the total tyrosine + tryptophan content was calculated (Bencze and Schmid, 1957) to be 7.2 per cent for A and 6.8 per cent for B. Using the average of these values for total content and the indicated ratio of tyrosine to tryptophan (0.6) it was possible to calculate that LDH with a molecular weight of 134,000 should contain 18 residues of tyrosine and 30 of tryptophan. The value for tyrosine is in excellent agreement with determinations made with an amino acid analyzer on total amino acid content.

Kinetic data. The substrate specificity of the 2 isozymes for α - γ -diketoacids and α -ketoacids was studied by means of the standard assay system in which 5 μ moles of the substrates were substituted for sodium lactate. The following acid substrates were tested: α - γ -diketovaleric, α - γ -diketocaproic,

α - γ -diketocapric, α -ketobutyric, α -ketovaleric, and α -ketocaproic. Although the absolute rates of oxidation of these substrates varied over a wide range, the 2 isozymes showed essentially the same relative catalytic behavior with all substrates.

Perhaps the most sensitive technique for revealing differences in enzymatic specificity is based upon the use of cofactor analogs (Kaplan *et al.*, 1960). The data in TABLE 3 shows the pyridine nucleotide cofactor specificity of each isozyme for TPN, DPN, and 3 analogs of DPN, all used in concentrations of $1.5 \times 10^{-2} M$. The rate of oxidation of sodium lactate when DPN was used as the cofactor provides the standard of comparison. It is evident that neither isozyme can use TPN. Both to the same degree utilize deamino-DPN more efficiently and acetyl-pyrimidine-DPN less efficiently than DPN. With thionicotinamide-DPN isozyme A is about 10 per cent more efficient than B, but this difference is probably not significant. With pyridine-3-

TABLE 3
PYRIDINE NUCLEOTIDE SPECIFICITY OF ISOZYMES A AND B*

Coenzyme analogue	Comparative enzyme activity	
	A	B
DPN	100	100
TPN	0	0
Deamino DPN	108	111
3-Acetylpyridine-*DPN	11	14
Pyridine-3-aldehyde-*DPN	44	34
Thionicotinamide-*DPN	75	83

* The standard assay system was used except that the indicated pyridine nucleotide cofactors were substituted for DPN at equivalent concentrations. Enzyme activity with each analogue is compared to the rate of enzyme activity with DPN taken as 100 per cent.

aldehyde-DPN the difference between A and B in rate of oxidation is 22 per cent—possibly on the borderline of significance.

The Michaelis-Menten constants for lactate and pyruvate were estimated for both isozymes from the linear plot of the initial reaction rates with the standard assay system according to the method of Lineweaver and Burk (1934). Again these two isozymes proved to be remarkably similar. When DPN and lactate were used, the K_m values for lactate were $1.5 \times 10^{-3} M$ for A and $1.7 \times 10^{-3} M$ for B. The K_m for pyruvate, when DPNH was used, was $5.0 \times 10^{-5} M$ for A and $5.4 \times 10^{-5} M$ for B.

In order to test the relative stability of the isozymes at different temperatures, small aliquots of the 2 fractions were heated for 3 min. in a water bath at several temperatures between 40° and 70° C. Essentially the same rates of inactivation occurred on preincubation at 60° C. and above.

Yet another test for enzymatic differences is sensitivity to chemical inhibitors. Oxamic acid is such an inhibitor of lactate dehydrogenase (Hakala *et al.*, 1953). Both isozymes proved to be equally sensitive to this inhibitor, being inhibited 80 per cent by $1 \times 10^{-3} M$ concentrations of oxamic acid.

Sulfhydryl groups are apparently important in maintaining the enzymatic activity of LDH. In the presence of $8 \times 10^{-4} M$ PCMB isozyme A was inhibited 41 per cent and B 43 per cent.

Discussion

It is evident from this investigation that the two most rapidly migrating isozymes of beef heart lactate dehydrogenase are remarkably alike; nevertheless the small differences that serve to distinguish them are based on very stable properties. No evidence for interconvertibility has been obtained. Essentially the same proportions of isozymes are observed at each step of the purification procedure from the crude homogenate to the final crystalline product. The resolution of the crystalline enzyme into its constituent isozymes is the same in the three systems used: starch gel, cellulose powder, and moving boundary electrophoresis. The variety of tests to which these isozymes have been put rules out preparative artifacts as a source of their origin. The single important distinction so far observed between them is the difference in charge. From an examination of a titration curve of each isozyme in the region of the isoelectric point (pH 4 to pH 5) it is evident that they differ by about 7 charges that are titrated per molecule over this range. This observation, together with the fact that the isoelectric points of the 2 isozymes differ by 0.3 pH units, implies that the isozymes differ by 2 or 3 charged groups, the A isozyme being the more negatively charged. This difference in charge could be achieved in several ways, perhaps most simply by amidation of carboxyl groups on glutamic or aspartic acid residues. Such slight changes would be difficult if not impossible to detect in analyses of total amino acid composition of such a large molecule as LDH. In fact our preliminary investigation of the primary structure of LDH isozymes in terms of amino acid composition has not so far revealed any differences. The data are not yet complete, but they do demonstrate the great similarity of these 2 particular isozymes and render unlikely any extensive difference in primary structure.

An examination of molecular properties based on secondary and tertiary structure has likewise failed to reveal conspicuous differences, but this aspect of the investigation is far from completed. Measurements of the specific rotation at 10 different wave lengths for each isozyme revealed only slight differences of doubtful significance. However, more extensive measurements of the optical rotation as a function of temperature, particularly in the region of denaturation, are now in progress, and these should provide a more sensitive test for slight differences in tertiary structure. Immunochemical properties depend in part upon the tertiary structure of the antigen and are frequently very sensitive expressions of slight changes in molecular configuration. In our hands, however, antisera prepared against isozyme A gave a reaction of identity in agar gel diffusion plates with isozyme B. Thus no antigenic difference was demonstrated.

The great similarity of the two particular isozymes studied in this investigation should not be taken as generally representative of all isozymes. We have chosen to study the two most closely related isozymes from a single tissue, but it is obvious that other isozymes of LDH differ to a much greater

extent in electrophoretic mobility, and this greater difference in mobility (or charge) should be reflected in other properties of the molecule as well. In fact, other investigators have already demonstrated conspicuous differences between certain isozymes of LDH, several of which are reported at this conference. Kaplan *et al.* (1960) have previously shown differences in analog specificity between LDH preparations from different tissues of the same animal. Although we now know that these preparations were mixtures of isozymes in different proportions, still any net differences between the different preparations must reflect differences between individual isozymes. Plagemann *et al.* (1960) have also shown differences in immunochemical behavior and in kinetic properties among the LDH isozymes of rabbit tissues and of human tissues. However, differences in isozymic properties cannot be fully appreciated until they can be related to molecular structure. At least five kinds of molecular changes may be invoked to produce different though closely related isozymes: small changes in amino acid sequence, amidation of carboxyl groups, conjugation with small molecules, polymerization, and changes in tertiary structure achieved by folding the same primary structure in different ways. All of these possibilities have served to explain with varying degrees of experimental support the existence of numerous enzymes in isozymic forms.

The existence of multiple molecular forms of enzymes is a tribute to the versatility of the cell in devising molecular configurations appropriate to the most specialized metabolic requirements. From the point of view of gene-controlled protein synthesis isozymes are rather perplexing. The report by S. L. Allen (1960) of allelic genes controlling alternate groups of esterases in *Tetrahymena* strongly supports the thesis that isozymes are modifications of a single gene product. The final tailoring of the molecule would thus be the responsibility of cytoplasmic mechanisms emerging during the course of cellular differentiation. This interpretation does not preclude the possibility that polygenic control may also exist. The total isozymic repertory (for a single enzyme) of an organism may be divisible into groups, each under the control of a separate gene. Polygenic control will be clearly indicated wherever isozymes are found to differ in primary structure. Other distinctions are more plausibly attributed to modifications imposed secondarily upon a single basic gene product. Additional genetic evidence is urgently needed, and though we have searched for genetic differences in isozymic patterns of LDH among numerous inbred strains of mice, none have so far been found.

Whatever the genesis of isozymes, their ontogeny and characteristic distribution imply physiological significance. The cytochemical investigation of J. M. Allen (1961) reveals that certain isozymes of LDH are distributed in characteristic locations within the cell and that they differ somewhat in enzymatic specificity. Likewise the two principal isozymes of malate dehydrogenase are found localized in the mitochondria and in the supernatant cytoplasm, respectively, after centrifugation (Thorne, 1960). The distribution of each isozyme to characteristic positions within the cell would enable them to perform distinctive metabolic roles even though their enzymatic properties were very similar. The work of Stadtman *et al.* (1961) on aspartokinase of

Escherichia coli provides the most engaging model of a useful role for isozymes. These aspartokinase isozymes were shown to be subject to different feedback controls which had the effect of maintaining useful concentrations of enzymatic activity to provide products for two quite distinct metabolic pathways.

Summary

Several physicochemical properties of purified crystalline preparations of two separated isozymes from beef heart lactate dehydrogenase have been investigated and measured. The only distinctive characteristic found is a difference in charge equivalent to two or three carboxyl groups. All other measurements suggested a remarkable similarity between these two particular isozymes.

The significance of isozymes was discussed with reference to protein structure, gene controlled protein specificity, intracellular localization, and distinctive metabolic activity.

References

- ALLEN, J. M. 1961. Multiple forms of lactic dehydrogenase in tissues of the mouse, their specificity and responses to altered physiological conditions. *Ann. N. Y. Acad. Sci.* **94** (3).
- ALLEN, S. L. 1960. Inherited variations in the esterases of *Tetrahymena*. *Genetics*. **45**: 1051-1070.
- ANDERSON, B. M., C. J. CIOTTI & N. O. KAPLAN. 1959. Chemical properties of 3-substituted pyridine analogues of diphosphopyridine nucleotide. *J. Biol. Chem.* **234**: 1219-1225.
- BENCZE, W. L. & K. SCHMID. 1957. Determination of tyrosine and tryptophane in proteins. *Anal. Chem.* **29**: 1193-1196.
- CALVIN, A. L. 1958. Simple microdetermination of kjeldahl nitrogen in biological material. *Anal. Chem.* **30**: 1692-1694.
- FLODIN, P. & D. W. KUPKE. 1956. Zone electrophoresis on cellulose columns. *Biochim. et Biophys. Acta*. **21**: 368-376.
- HAKALA, M. T., A. J. GLAD & G. W. SCHWERT. 1953. Kinetics and specificity of lactic dehydrogenase. *Federation Proc.* **12**: 213.
- KAPLAN, N. O., M. M. CIOTTI, M. HAMOLSKY & R. E. BIEBER. 1960. Molecular heterogeneity and evolution of enzymes. *Science*. **131**: 392-397.
- LINEWEAVER, H. & D. BURK. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**: 658-666.
- MARKERT, C. L. & F. MØLLER. 1959. Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci.* **45**: 753-763.
- NACHLAS, M. M., S. I. MARGULIES, J. B. GOLDBERG & A. M. SELIGMAN. 1960. The determination of lactic dehydrogenase with a tetrazolium salt. *Anal. Biochem.* **1**: 317-326.
- NEILANDS, J. B. 1952. Studies on lactic dehydrogenase of heart—purity, kinetics, and equilibria. *J. Biol. Chem.* **199**: 373-381.
- NEILANDS, J. B. 1955. Lactic dehydrogenase of heart muscle. *In Methods in Enzymology*. **I**: 449-454. S. P. Colowick and N. O. Kaplan, Eds. Academic Press. New York, N.Y.
- PICKELS, E. G., W. F. HARRINGTON & H. K. SCHACHMAN. 1952. An ultracentrifuge cell for producing boundaries synthetically by a layering technique. *Proc. Natl. Acad. Sci.* **38**: 943-948.
- PLAGEMANN, P. G. W., K. F. GREGORY & F. WRÓBLEWSKI. 1960. The electrophoretically distinct forms of mammalian lactic dehydrogenase. **I**. Distribution of lactic dehydrogenase in rabbit and human tissues. *J. Biol. Chem.* **235**: 2282-2287.
- Ibid.* The electrophoretically distinct forms of mammalian lactic dehydrogenase. **II**. Properties and interrelationships of rabbit and human lactic dehydrogenase isozymes. *J. Biol. Chem.* **235**: 2288-2293.
- SCHACHMAN, H. K. 1957. Ultracentrifugation, diffusion, and viscometry. *In Methods in Enzymology*. **IV**: 32-103. Colowick, S. P. and N. O. Kaplan, Eds. Academic Press. New York, N.Y.

- STADTMAN, E. R., G. N. COHEN & G. LEBRAS. 1961. Selective feedback inhibition and repression of two aspartokinases in the metabolism of *Escherichia coli*. Ann. N. Y. Acad. Sci. **94** (3).
- STRAUB, F. B. 1940. Crystalline lactic dehydrogenase from heart muscle. Biochem. J. **34**: 483-486.
- SVEDBERG, T. & K. O. PEDERSEN. 1940. The Ultracentrifuge. Clarendon Press. Oxford, England.
- THORNE, C. J. R. 1960. Characterization of two malic dehydrogenases from rat liver. Biochem. Biophys. Acta. **42**: 175-176.

CHEMICAL DIFFERENCES BETWEEN MULTIPLE FORMS OF LACTIC ACID DEHYDROGENASES*

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In 1950, Meister¹ observed in the Tiselius electrophoresis of crystalline beef-heart lactic dehydrogenase (LDH) a faster-moving component (A), to which he accounted the whole enzymatic activity, and also a slower-moving one that amounted to about 15 per cent. Two years later, Neilands² noticed that this second protein (called by him component C) also possesses LDH activity. In 1956 we started³ an extensive investigation of physical and biochemical differences between LDHs, after having seen that many preparations of LDH from different origins can be resolved into several, up to five, proteins with the activity of LDH, by electrophoresis on paper or in a layer block of starch. Later⁴ it was found that minimal amounts of enzyme mixtures can be quickly separated in the electrical field on a foil of acetyl cellulose. The single enzymes then can be eluted and evaluated quantitatively in an optical test conducted to determine the rate of hydrogenation of pyruvic acid by DPNH. Thus we succeeded in showing that nearly all organs of an animal contain the same number of LDH proteins, usually five, sometimes even more. The corresponding components have the same migration rate but are distributed in a way characteristic for each organ. The distribution patterns of LDHs of several rat tissues⁵ are presented in FIGURE 1.

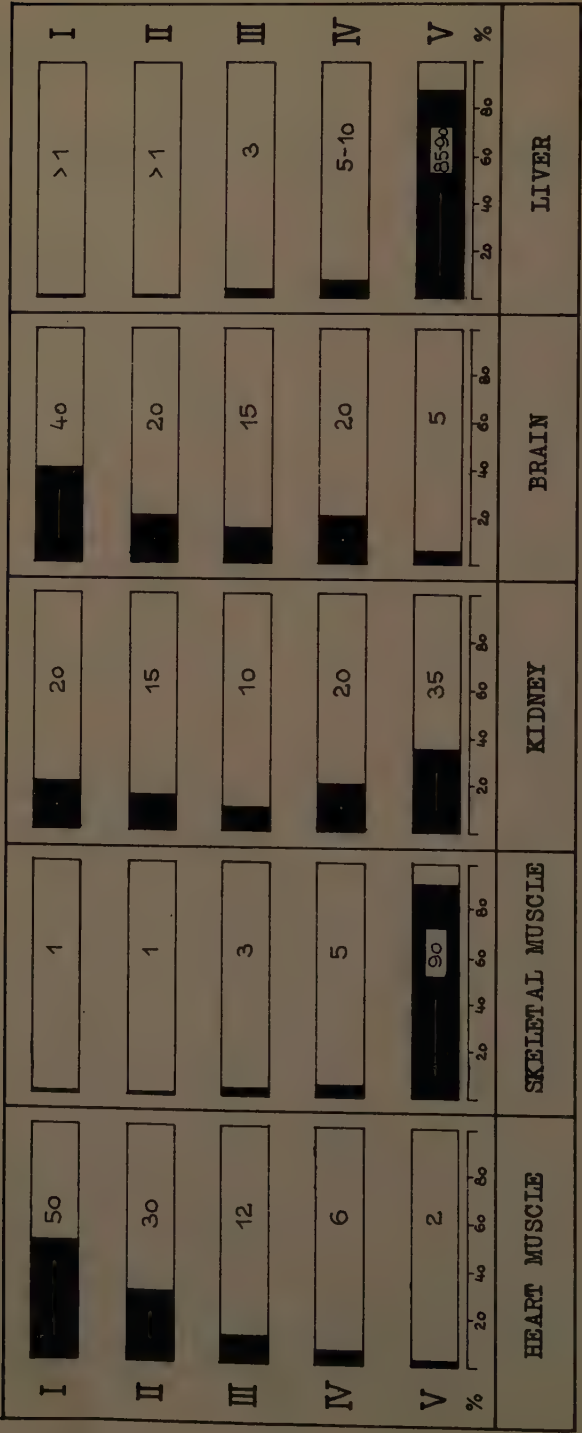
In the following paragraphs we shall deal with proteins I, II, III, and IV† of heart muscle, and protein V of skeletal muscle and liver of the rat. In addition to these enzymes, bands I of pig heart and V of rabbit skeletal muscle were checked. All of them were prepared as described in earlier papers. The preparative resolution of heart-muscle components was achieved by zone electrophoresis in a starch layer block, the different bands being marked by a reprint on filter paper. The single proteins then were eluted from glass powder into which they had been directed by electrophoresis.⁶

The similar migration in paper electrophoresis and the same inhibition by sulfite ions of equally moving proteins strongly led to the suggestion that these were identical in spite of the tissue of origin. As we found some years ago, however,⁴ the analogous proteins No. I of heart, kidney, and brain of the rat exhibit different optimal pyruvate concentrations; the differences, however, were not very striking (6×10^{-7} , 9×10^{-7} , and 1×10^{-6} m. respectively). Therefore only the chemical analysis of different enzymes could prove either their identity or heterogeneity. We have now isolated the above-mentioned enzymes in extreme pure form and analyzed their amino-acid composition. In so far as we know, all of them have the same molecular weight, 130,000. The

* This paper is No. 6 in a series, "Heterogeneity of Lactic Dehydrogenases." Paper No. 5 is Wieland *et al.*³

† Contrary to Plagemann *et al.*,⁹ the number "I" is attributed to the fastest moving band, because no faster one has been detected until now although, sometimes, further bands appear beyond No. V, which are designated consecutively as, for example, VI and VII.

⊕



⊖

FIGURE 1. Distribution pattern of LDH proteins in several organs of the rat.

analyses were carried out chromatographically after 72 hours' hydrolysis with 6 n HCl at 110°, using a Moore and Stein technique devised by R. Weber in the laboratory of M. Brenner in Basel, Switzerland. The results are cited in TABLE 1.

The amino-acid composition of rat-liver LDH already had been determined microbiologically in 1953 by Vestling and his co-workers.⁷ Their results, which agree rather well with ours on some points, are also indicated in TABLE 1, and are shown comparatively in FIGURE 2.

Before the chromatographic analyses of amino acids, the same LDHs were degraded by trypsin, and the tryptic peptides were separated by paper electrophoresis at high voltage. Although the results of this work have been pub-

TABLE 1
NUMBERS OF AMINO ACID MOLECULES PER MOLE (130,000) OF SEVERAL
LACTIC DEHYDROGENASES

	Rat liver					Rat muscle				Rat-heart proteins				Pig-heart protein I		Rabbit muscle
	Lit. ⁷	1	2	3	4	1	2	3	4	I	II	III	IV	1	2	V
Lys.	90	98	100	100	100	101	99	101	103	89	88	89	84	87	86	98
His.	17	23	24	25	25	22	21	22	23	26	26	27	29	25	27	37
Arg.	40	38	37	39	40	38	38	38	38	31	35	39	53	28	32	35
Asp.	117	117	117	115	113	119	117	118	118	138	127	123	109	125	125	108
Glu.	80	110	107	106	110	106	105	105	104	103	110	110	120	115	116	106
Ala.		76	75	74	79	73	72	71	72	75	82	83	93	70	70	76
Gly.	98	91	91	90	95	91	91	91	90	85	90	94	102	87	87	94
Ileu.	85	82	85	83	79	87	85	83	84	81	78	75	64	80	76	79
Leu.	133	127	129	127	124	134	131	131	130	128	123	118	104	128	125	130
Met.	21	26	25	25	25	25	26	25	25	32	28	27	23	33	30	32
Phe.	24	29	28	28	30	25	26	25	26	20	28	32	41	19	21	26
Pro.	68	49	46	51	51	51	47	46	44	42	47	51	48	45	47	38
Ser.	93	91	86	87	88		94	92	88	95	92	91	79	86	92	85
Thr.	35	46	45	44	47		42	43	43	53	55	55	63	55	57	47
Tyr.	20	27	27	26	27	25	26	25	26	24	25	27	31	25	27	26
Val.	140	116	126	124	121	132	129	130	131	134	117	113	90	139	132	126

lished recently,⁸ we should like to demonstrate the schematic drawings of some pherograms. It may be seen that all LDHs thus far investigated are more or less different. The extent of the difference, however, cannot be estimated from the one-dimensional pherograms.

FIGURE 2 shows the separation of tryptic peptides of LDHs from rat heart (*left*) and pig heart (*right*). They are of a rather surprising similarity. In FIGURE 3 the numbers of some amino acids per molecule of the same enzymes are diagrammatically represented. Here a great similarity also is evident, although there exist distinct differences, for example in aspartic and glutamic acid as is to be expected in the case of different species.

Greater differences exist in the electrophoretic peptide patterns of LDHs derived from skeletal muscle of rabbit (*left*) and rat (*right*) in FIGURE 4. The analyses of their amino acids reveal that both these enzymes are proteins that strongly differ in their primary structure (see TABLE 1).

Next let us consider the amino-acid composition of LDHs from two different organs of one animal namely of skeletal muscle and liver of rat (FIGURE 5). Neither migrates in the electric field, and neither is distinguishable in other respects, except as regards their maximal turnover numbers (67,000 and 45,000).⁴ Here the chemical differences are extremely small, but significant

Pherograms at pH 6.5
of tryptic peptides
of LDHs from
RAT HEART PIG HEART

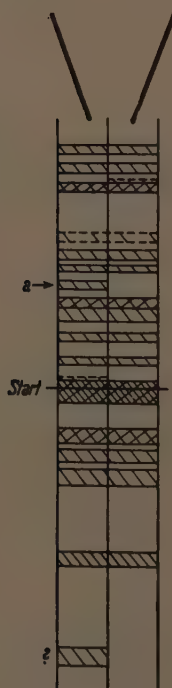


FIGURE 2.

and reliable on account of four analyses of two different samples. Accordingly, we are facing an organ specificity of LDH synthesis that can be demonstrated even more clearly, at least in the rat, comparing skeletal LDH V with heart LDH I (FIGURE 3). They differ not only in the ratio of acidic to basic side chains, which the electrophoretic mobility depends on, but also in the numbers of nearly all of the amino acids. Previously it was found by electrophoretic comparison that the tryptic peptides exhibited striking differences too (FIGURE 6).

Finally the amino acids of rat-heart isoenzymes I, II, III, and IV were determined. Only very small differences had been observed in the pattern of their tryptic digests (FIGURE 7). Therefore the assumption was made⁸ that the decreasing mobility of proteins I to V could arise from an increasing number of carboxamide groups attached to an identical original protein. To our

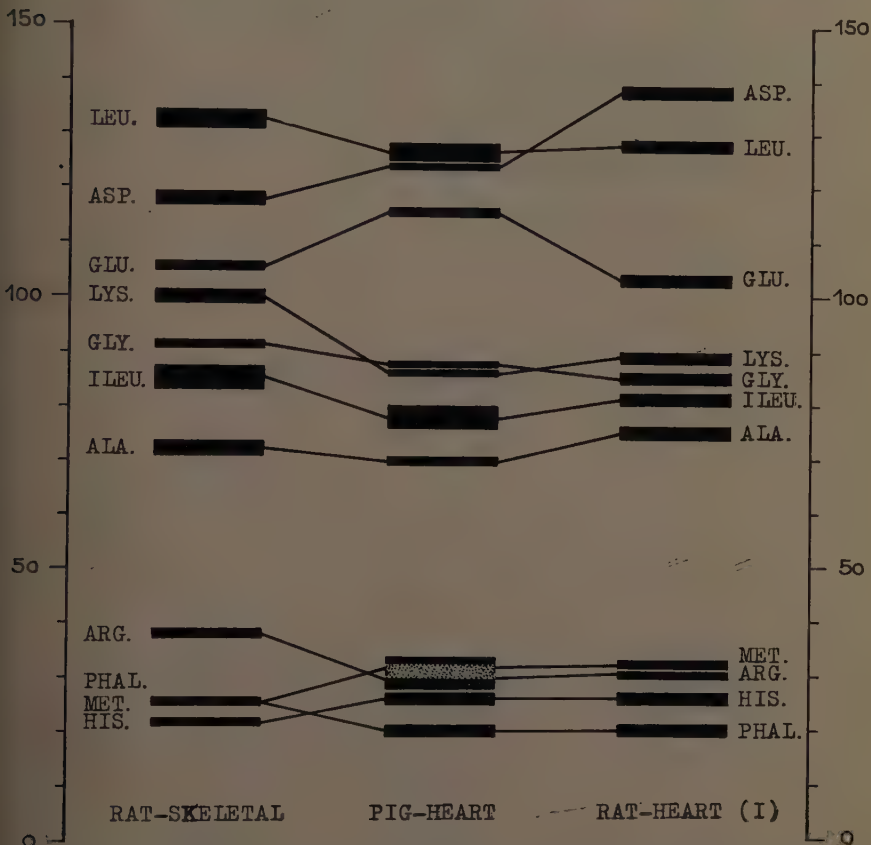


FIGURE 3. Differences between the amino-acid composition of rat-skeletal LDH, pig-heart LDH, and rat-heart LDH. Ordinate: numbers of amino acids per mole of LDH (130,000).

greatest surprise, however, the analyses of the individual LDHs I, II, III, and IV disclosed that the ratios of their amino acids differ in an unforeseen manner (FIGURE 8). It seems that four, probably five, quite different enzymes of the same action (homotropic enzymes) are synthesized in one organ, presumably even in one cell. Whether their amino acids are filed in completely different sequences, or according to certain regularities, only will be recognized by analysis of primary protein structures.

In conclusion, let us discuss some connections between composition and

electrophoretic behavior of our proteins. As was shown in FIGURE 1 and, as is generally known, the LDH proteins No. V do not possess electrophoretic mobility at pH 8.6. Because at this pH , the imidazole nucleus of histidine is unprotonated, the sum of the positive arginine and lysine side chains should equal to

Pherograms at pH 6.5
of tryptic peptides
of LDHs from

RABBIT RAT
MUSCLE MUSCLE

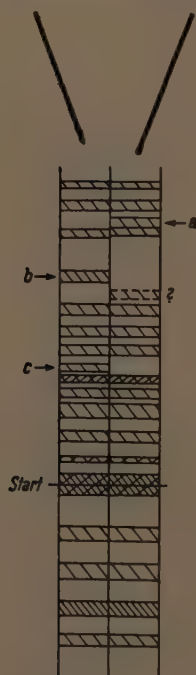


FIGURE 4.

the negative β -aspartyl and γ -glutamyl side groups. According to the results of Weber (TABLE 1), in LDH from rat skeletal muscle and rat liver, the sum of $+$ charges is 138 and the sum of $-$ charges 223 and 225; that would mean a net charge of about 90 negative units. All of the LDH proteins, however, contain amide groups, whose quantity not exactly comes out after hydrolysis with strong acids, because ammonia also is formed by decomposition of serine, threonine, tryptophane, and other amino acids. The amount of ammonia was

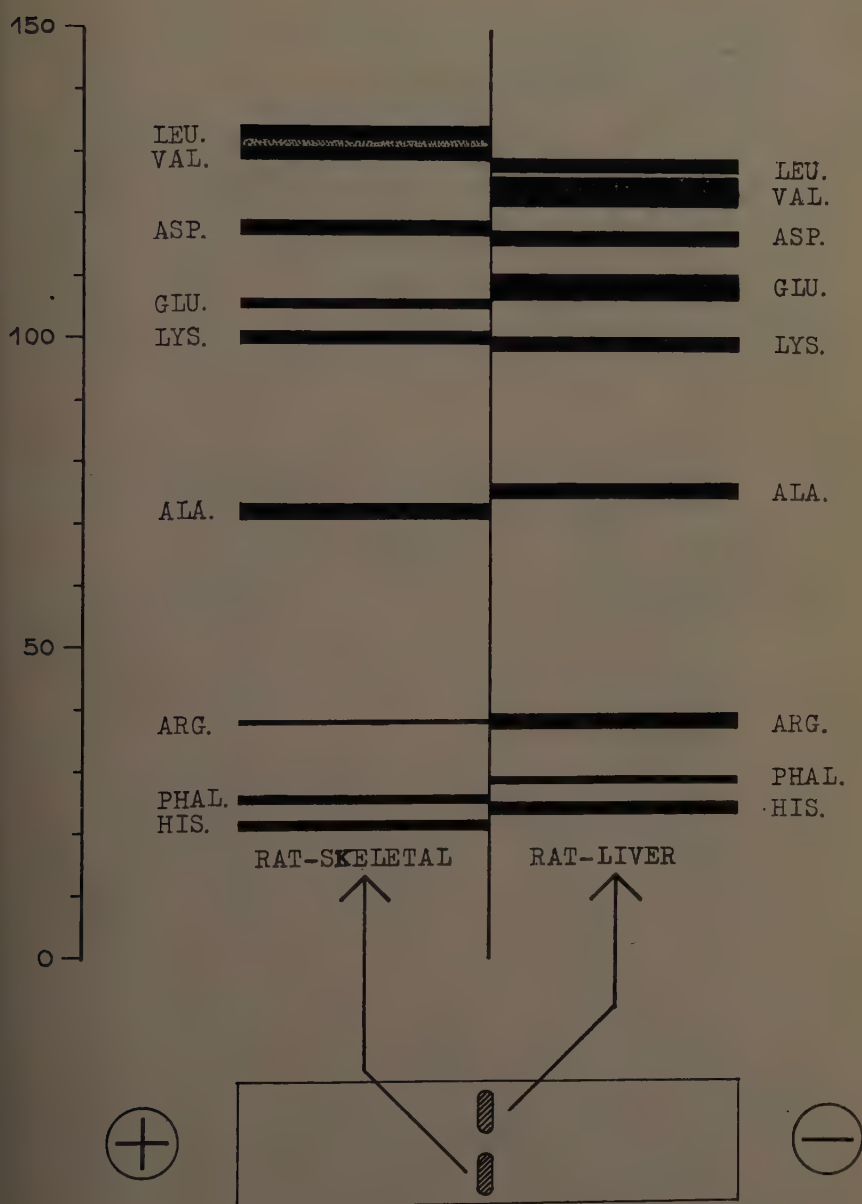


FIGURE 5. Differences between rat-skeletal LDH and rat-liver LDH.

rather constant in all enzyme samples, about 150 moles per mole of protein. From these, one can contribute about 60 moles to decomposition and 90 to amide origin. Consequently the net charge of the nonmobile LDHs at pH 8.6 would be zero. An analogous calculation gives the results shown in TABLE 2.

The astonishingly good agreement between mobility and calculated net charge impossibly can be only by chance, but seems to constitute further proof

**Pherograms at pH 6.5
of tryptic peptides
of LDHs from
RAT HEART (I) RAT
MUSCLE**

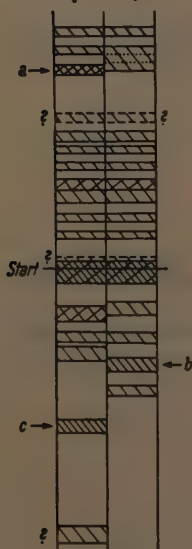


FIGURE 6.

**Pherograms at pH 6.5
of tryptic peptides
of RAT HEART LDHs
I II III**

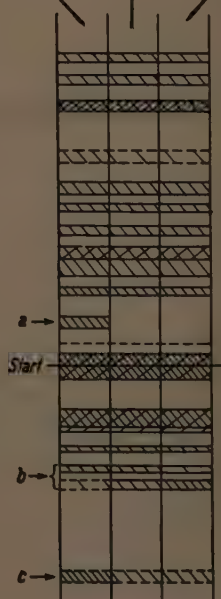


FIGURE 7.

of the correctness of our amino-acid determinations. From our investigations, which are being continued, it is evident that the mammals do not contain only one LDH, as was believed earlier but—as the example of rat shows—contain at least seven different LDH proteins, presumably even many more. The biological meaning of this fact is not at all clear to us today.

Acknowledgments

We are grateful to W. Gruber, K. Rajewsky, and H. L. Rettig for their kind assistance.

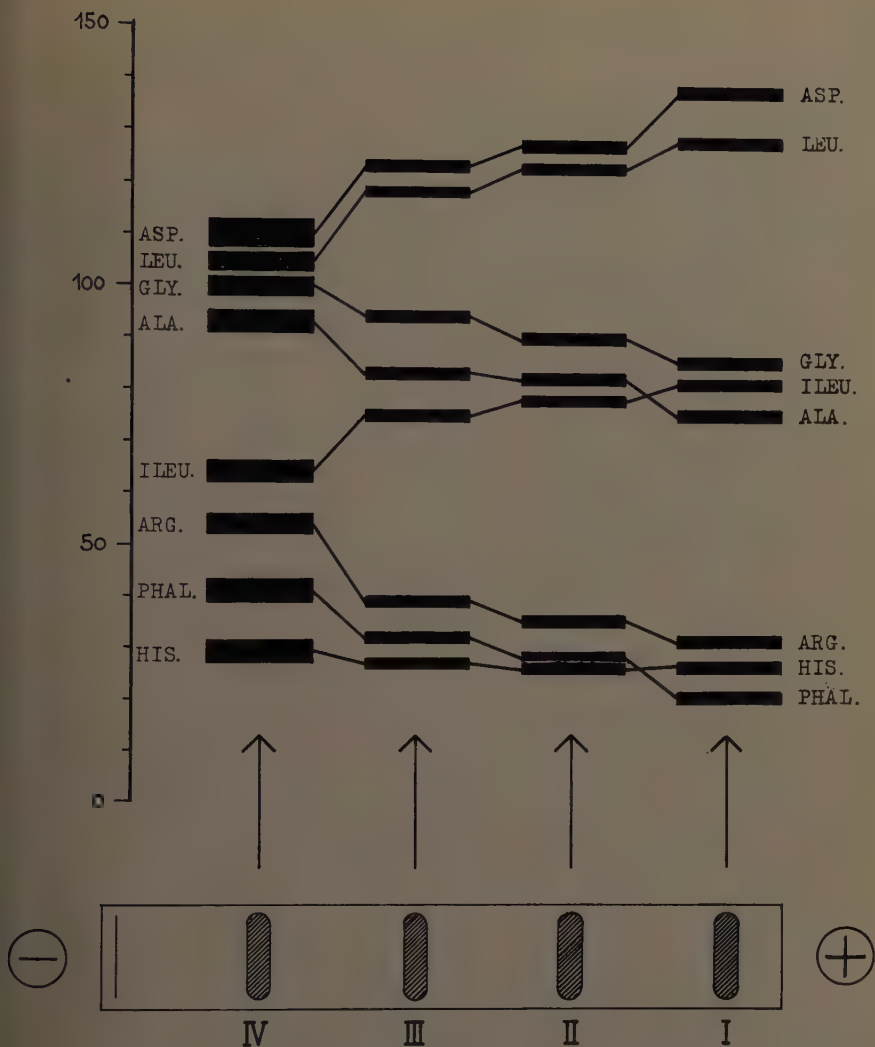


FIGURE 8. Some clear differences between amino-acid composition of rat-heart LDH components I to IV.

TABLE 2

	Lactate dehydrogenase from:						
	Rat						Pig heart
	Skeletal	Liver	Heart				
			I	II	III	IV	
⊕ (Arg. + lys.)	138	138	120	123	128	137	117
⊖ (Glu. + asp.) - 90	133	135	151	147	143	140	150
Difference	+5	+3	-31	-24	-15	-3	-33
Mobility to anode (arbitrary units)	0	0	100	75	50	25	100

References

1. MEISTER, A. 1950. Reduction of α , γ -diketo and α -keto acids catalized by muscle preparations and by crystalline lactic dehydrogenase. *J. Biol. Chem.* **184**: 117-129.
2. NEILANDS, J. B. 1952. Studies on lactic dehydrogenase of heart I. Purity, kinetics, and equilibria. *J. Biol. Chem.* **199**: 373-381.
3. WIELAND, TH. & G. PFLEIDERER. 1957. Nachweis der Heterogenität von Milchsäuredehydrogenasen verschiedenen Ursprungs durch Trägerelektrophorese. *Biochem. Z.* **329**: 112-116.
4. PFLEIDERER, G. & D. JECKEL. 1957. Individuelle Milchsäure-dehydrogenasen bei verschiedenen Säugetieren. *Biochem. Z.* **329**: 370-380.
5. WIELAND, TH., G. PFLEIDERER & F. ORTANDERL. 1959. Vergleiche der Milchsäuredehydrogenasen aus verschiedenen Rattenorganen. *Biochem. Z.* **331**: 103-109.
6. WIELAND, TH., G. PFLEIDERER, I. HAUPT & W. WÖRNER. 1959. Quantitative Ermittlung einiger Enzymverteilungsmuster. Vergleichende Betrachtung bei verschiedenen Wirbeltierklassen. *Biochem. Z.* **332**: 1-10.
7. WIELAND, TH., G. PFLEIDERER & H. L. RETTIG. 1958. Verfahren zur Wiedergewinnung von Proteinen aus Trägerschicht-Elektropherogrammen. *Angew. Chem.* **70**: 341-350.
8. GIBSON, D. M., E. O. DAVISSON, B. K. BACHHAWAT, B. R. RAY & C. S. VESTLING. 1953. Rat liver lactic dehydrogenase I. Isolation and chemical properties of the crystalline enzyme. *J. Biol. Chem.* **203**: 397-409.
9. WIELAND, TH., G. PFLEIDERER & K. RAJEWSKY. 1960. Verschiedenheit der Milchsäure dehydrogenasen V. Papierelektrophoretischer Vergleich der tryptischen Spaltprodukte. *Z. Naturforsch.* **15b**: 434-436.
10. PLAGEMAN, P. G. W., K. F. GREGORY & F. WRÓBLEWSKI. 1960. The electrophoretically distinct forms of mammalian lactic dehydrogenase I. Distribution of lactic dehydrogenases in rabbit and human tissues. *J. Biol. Chem.* **235**: 2282.

Discussion

MAYO UZIEL (*Department of Biochemistry, Tufts University School of Medicine, Boston, Mass.*): From the criteria discussed in previous papers it seems clear that the several ribonuclease (RNase) components present in pancreas are isozymes. E. Bartos and myself have performed a comparative study of these isozymes isolated from calf pancreas and guinea pig pancreas. We separated the various subcellular fractions after homogenization and analyzed each by chromatography on IRC-50.

Large differences were observed with this type of analysis. On comparison of the chromatographic patterns of total extracts of the tissue, the calf pancreas contained about 90 per cent as RNase A (the more highly adsorbed component); whereas the guinea pig pancreas has only 65 per cent. On analysis, the subcellular fractions of the calf pancreas gave the same pattern: that is, 90 per cent RNase A and 10 per cent RNase B. On the other hand, the guinea pig pancreas subcellular fractions gave different patterns: the zymogen granules had about 80 per cent RNase A, the microsomes and ribonucleoprotein particles had about 75 per cent RNase A, while the supernatant fluid had only 55 per cent as RNase A.

Therefore, although there are discrete subcellular particles containing the enzyme, the mechanism of their formation is not necessarily dependent upon their location within the cell but on some more complex system, for example, the mechanism of their biosynthesis.

EVOLUTION AND DIFFERENTIATION OF DEHYDROGENASES*

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During the past eight years we have been carrying on investigations involving pyridine coenzyme analogues. We found that the analogues could be used to show the heterogeneity of enzymes catalyzing the same function. This paper summarizes some of our results obtained with the analogues and illustrates the use of the catalytic technique to determine molecular heterogeneity.

Types of Coenzyme Analogue Used

We have prepared analogues of diphosphopyridine nucleotides (DPN) and of triphosphopyridine nucleotides (TPN), which are substituted in the adenine and nicotinamide moieties. Both types of substitution are useful in studies on heterogeneity. TABLE 1 lists some of the pyridine analogues used for studies on heterogeneity, and TABLE 2 lists some of the analogues which are purine derivatives.

Alcohol Dehydrogenases

The yeast alcohol dehydrogenase molecule is very different from the horse liver alcohol dehydrogenase molecule, although both catalyze the oxidation of ethanol. The difference can be dramatically demonstrated by the reaction rates with a number of coenzyme analogues, as illustrated in TABLE 3. It is remarkable that certain analogues, which do not react with the crystalline yeast enzyme, react with the crystalline horse liver catalyst at a much faster rate than with the natural coenzyme. Hence the analogues clearly show that there is a big difference between the two enzymes. When such extreme differences are found with the analogue reactions, large differences are also found with respect to physical properties of the enzymes. It is known that the molecular weight of the yeast enzyme is approximately twice that of the liver enzyme. The yeast contains 4 moles of zinc per mole of enzyme and binds 4 moles of DPN; the liver catalyst has 2 moles of zinc and binds 2 moles of the coenzyme.¹ Ludwig Brand and Johannes Everse of our laboratory have found that the two alcohol dehydrogenases differ greatly in their response to denaturing agents.

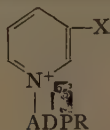
D and L Lactic Dehydrogenases

In *Lactobacillus plantarum* there exist two lactic dehydrogenases. One is specific for L(+) lactate and the second for D(−) lactate. These two enzymes are difficult to separate either by electrophoresis or by column chromatography. It has been possible to distinguish the two enzymes by their rates of reduction of DPN analogues.² This is shown in TABLE 4. Both enzymes are specific

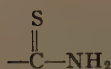
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for DPN and do not react with TPN. Both react with the 3-acetylpyridine DPN at a much faster rate than with DPN. The two dehydrogenases indicate that there are two distinct enzymes, since one enzyme reacts better with one group of analogues than the second does, and the second reacts at a faster rate

TABLE 1
PYRIDINE-SUBSTITUTED ANALOGUES



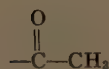
X



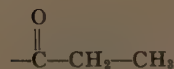
3-thionicotinamide-*DPN (TNDPN)



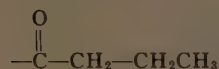
pyridine-3-aldehyde-*DPN (Py3AlDPN)



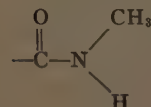
3-acetylpyridine-*DPN (APDPN)



3-ethylpyridyl ketone-*DPN (EPKDPN)



3-propylpyridyl ketone-*DPN (PPKDPN)



N-methyl nicotinamide-*DPN



5-methyl nicotinamide-*DPN†

† First prepared in our laboratory by P. Walter.

than the first with other analogues. It has been possible to show that the L(+) and D(-) lactic dehydrogenases of the *Lactobacillus* are different structurally. This has been done by immunochemical methods as well as by demonstrating marked differences in sensitivity to temperature. Furthermore, it has been found that the L(+) enzyme is inhibited by oxamate, whereas the D(-) enzyme is not. These data show that when differences in some properties of the enzymes are observed by means of the coenzyme analogues, differences in other properties of the enzymes are present.

Vic Glycol Dehydrogenases

Two DPN-requiring *vic* glycol dehydrogenases can be isolated from extracts of a variant of *Aerobacter aerogenes*. One of these two dehydrogenases appears when the cells are grown on a glycerol medium; the second enzyme arises when the organism is grown on a glucose medium.³ These two enzymes are physi-

TABLE 2
ADENINE-SUBSTITUTED ANALOGUES

Substitution	
	nicotinamide hypoxanthine dinucleotide (deamino DPN) (DeDPN)
	nicotinamide uracil dinucleotide† (uracil-*DPN)
	nicotinamide-1-(2-hydroxyethyl) adenine dinucleotide† (N ₁ EtoDPN)
	nicotinamide-6-(2-hydroxyethyl) purine dinucleotide‡ (N ₆ EtoDPN)

† First prepared in our laboratory by C. P. Fawcett.

‡ First prepared in our laboratory by H. Windmueller.

TABLE 3
COMPARISON OF REACTION RATES OF SOME ANALOGUES OF DPN WITH YEAST AND HORSE LIVER ALCOHOL DEHYDROGENASES

Coenzyme	Yeast ADH	Liver ADH
DPN	1	1
Deamino-*DPN	0.12	1.1
3-Acetylpyridine-*DPN	0.05	6.0
Pyridine-3-aldehyde-*DPN	<0.02	0.95
3-Thionicotinamide-*DPN	0.16	3.5
3-Benzoylpyridine-*DPN	0	0.31
Propyl pyridyl ketone-*DPN	<0.01	4.8
Uracil-*DPN	0.02	0.75

Ethanol (0.1 M) was used in all reaction mixtures.

cally inseparable. However, as indicated in TABLE 5, they show remarkably different reactions with the coenzyme analogues. Differences in stability and immunochemical characteristics, as well as in pH optima, show that the two enzymes are dissimilar. These studies with the *vic* glycol dehydrogenases demonstrate that closely related enzymes can have properties that make them difficult to distinguish by physical means, even though they are heterogeneous.

TABLE 4
PYRIDINE NUCLEOTIDE SPECIFICITY OF LACTIC DEHYDROGENASES
FROM *LACTOBACILLUS PLANTARUM*

Coenzyme or analogue	DPN rate taken as 100%	
	D(-)-LDH (%)	L(+)-LDH (%)
TPN	0	0
3-Acetylpyridine-*DPN	430	1300
Pyridine-3-aldehyde-*DPN	7	57
Thionicotinamide-*DPN	52	185
3-Benzoylpyridine-*DPN	215†	13†
Deamino DPN	60	0

† Rate for 55 min., whereas all other rates are for the 30- to 90-sec. interval.

TABLE 5
VIC GLYCOL DEHYDROGENASES FROM *AEROBACTER AEROGENES*†

	Glucose grown	Glycerol grown
	(relative rates)	
DPN	100	100
Deamino DPN	12	252
Acetylpyridine-*DPN	73	4
Pyridine-3-aldehyde-*DPN	55	20

† From Lamborg and Kaplan.³

Hexitol Phosphate Dehydrogenases

Maurice Liss, in collaboration with Susan Horwitz, has made in our laboratory another interesting use of the analogues in distinguishing properties of the dehydrogenases. They have been studying the mannitol and sorbitol phosphate dehydrogenases from *Aerobacter aerogenes*. The two hexitol phosphates are isomers and differ only in the configuration around one carbon atom. The dehydrogenases are specific, that is, one enzyme reacts only with the mannitol phosphate; the second, however, reacts with the sorbitol phosphate. In early studies it appeared that the activities of the two enzymes were due to different proteins, since it was found that their reactions with the coenzyme analogues differed considerably. TABLE 6 shows the strikingly big difference between the two enzymes in their reaction with the thionicotinamide-*DPN. The two hexitol phosphate dehydrogenases have now been extensively purified. It is

of interest that the mannitol phosphate dehydrogenase has a molecular weight of approximately 30,000; the sorbitol phosphate dehydrogenase, however, appears to have a molecular weight 3 to 4 times as great. The two enzymes also have been found to be considerably different in their heat stability, in their requirements for sulfhydryl groups, as well as in manifestations of some slight difference in their electrophoretic mobilities.⁴ Studies such as the above give us the impression that when there is a great difference in reactivity with the analogues, one will find considerable variation in the physical properties of the enzyme.

Substrate Inhibition of Dehydrogenases

During our first studies with the coenzyme analogues we observed that several of the analogues reacted differently to beef and to rabbit skeletal muscle lactic dehydrogenases.⁵ These studies indicated to us that the two enzymes were heterogeneous. We also noted that the dehydrogenases as a group showed

TABLE 6
AEROBACTER HEXITOL PHOSPHATE DEHYDROGENASES

	Mannitol 1-phosphate dehydrogenases	Sorbitol 6-phosphate dehydrogenases
	(Relative rates)	
DPN	100	100
6-(2-hydroxyethyl amino)purine-*DPN	75	7
Thionicotinamide-*DPN	<1	100
Deamino DPN	70	<1

characteristic substrate inhibition,^{6,8} and that the Michaelis constant depended on the type of coenzyme employed. FIGURE 1 illustrates this. It shows that the affinity of the substrate for the enzyme is determined by the coenzyme used. The difference between the analogues, such as the reduced ethylpyridyl ketone and the 6,2-hydroxyethyl purine derivative, is most striking. With low levels of substrate, one analogue shows a rapid reaction; with increasing levels of pyruvate, marked substrate inhibition is shown. The reverse is seen with the other analogue where there is negligible reaction with low levels of substrate. As the substrate concentration is increased, however, the rate accelerates. With TPNH and low concentrations of pyruvate, the reaction rate with beef heart dehydrogenase is practically negligible (see FIGURE 1); only with high concentrations of pyruvate is there significant activity with the reduced TPN.⁷ It is important to note that the inhibition with the substrate varies with the dinucleotide coenzyme used. Elsewhere we have discussed the significance of the variation in substrate saturation with different coenzymes with regard to the ternary complex as intermediates in dehydrogenase reactions.^{6,8} It should be emphasized that substrate saturation occurs not only with lactic dehydrogenases, but also with malic, alcohol, glutamic dehydrogenases, and other enzymes of this general type.

Heterogeneity of Lactic Dehydrogenases

We have surveyed the lactic dehydrogenases of different tissues as well as those from different animals.⁷ In these studies we have used the coenzyme analogues together with three concentrations of each substrate. The concentrations of substrates used in lactic and malic dehydrogenase studies are summarized in TABLE 7. When referring in the tables to the concentration of the substrates used, we have made the following designations: DPN(1), DPN(2), DPN(3), and continuing upward. This indicates that with DPN(1) the low concentration of lactate or malate was used. DPN(2) refers to conditions

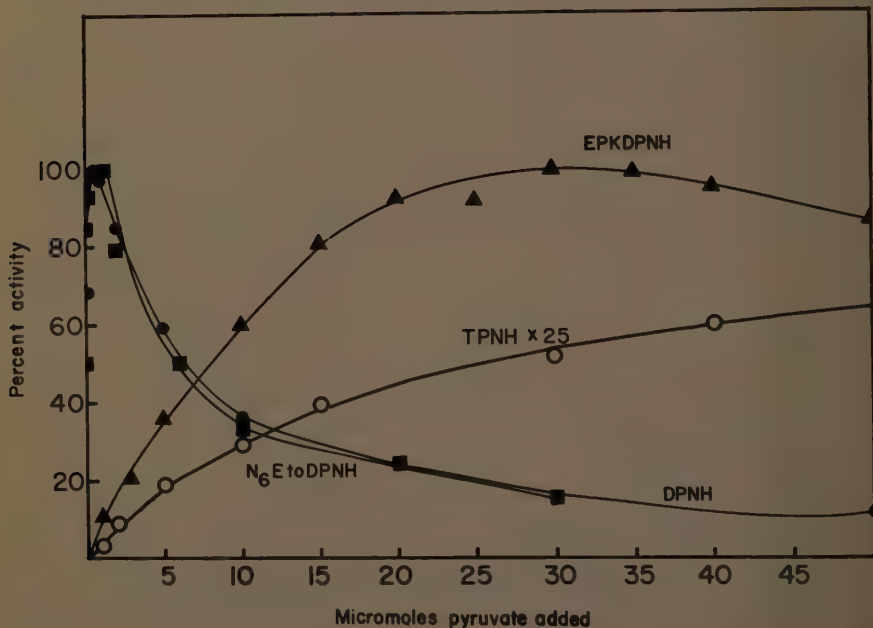


FIGURE 1. Effect of pyruvate concentration on the reaction rates of beef heart lactic dehydrogenase with different reduced pyridine nucleotides. With TPNH, 25 times as much enzyme was used as with the other nucleotides. The pH of the reaction was 7.5.

wherein the middle concentration of oxaloacetate or pyruvate was employed. In the case of utilization of analogues as coenzymes, the same designations are maintained. For example, the designation APDPN(3) refers to experiments wherein the highest level of lactate or malate was used with acetylpyridine-DPN. The substrate saturation and substrate inhibition properties of an enzyme are most useful for the recognition of heterogeneity. One can distinguish some differences in closely related enzymes by carefully studying their affinity for the natural coenzyme; in general, however, the analogues appear to magnify the differences.

In surveying the characteristics of the dehydrogenases, we usually used crude extracts. However, after purifying a number of these enzymes, we found no significant differences in the reaction spectra of the analogues with the crude

and purified enzymes. We have found great differences in the analogue reaction with respect to the lactic dehydrogenases from different sources. FIGURE 2 illustrates these differences by comparing the saturation curves of lobster heart lactic dehydrogenase with the beef heart catalyst. The low affinity of the lobster enzyme for the natural coenzyme, in comparison with the acetylpyridine DPN, is indicative of the great difference between the two enzymes.

TABLE 7

SUBSTRATE CONCENTRATIONS USED IN THE LACTIC AND MALIC DEHYDROGENASE STUDIES

	Final molar concentrations		
	(1)	(2)	(3)
DL-Lactate	1.3×10^{-2}	1×10^{-1}	2×10^{-1}
Pyruvate	3×10^{-4}	3×10^{-3}	1×10^{-2}
L-Malate	6×10^{-3}	5×10^{-2}	1×10^{-1}
Oxaloacetate	2.6×10^{-4}	2.6×10^{-3}	$8 \times 10^{-3*}$

* In extracts where there may be high levels of lactic dehydrogenase, and where a high concentration of oxaloacetate is used, it is necessary to add oxamate because there may be significant amounts of pyruvate present in most preparations of oxaloacetate.

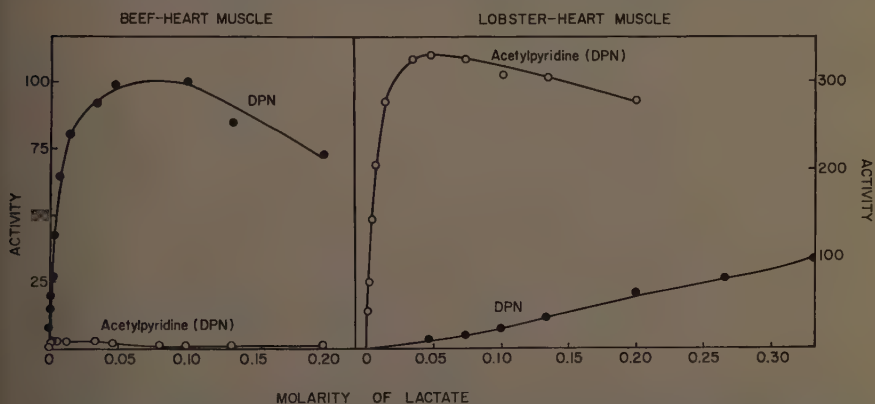


FIGURE 2. Comparison of the effect of pyruvate concentration on the reaction rates of beef heart and lobster heart lactic dehydrogenases. Reproduced by permission from *Science*.⁷

It is noteworthy that under normal conditions for assaying lactic dehydrogenases with lactate, one would assume that there is almost no enzyme present in the lobster heart. As indicated by the reaction with the acetylpyridine DPN, however, appreciable amounts of this enzyme appear to be present.

In general, we found that closely related species have closely related enzymes. For example, mammalian heart enzymes seem to have characteristics that appear to be quite common for all the enzymes of the group. However, the analogue reactivities indicated differences in the individual tissues of the mammals. As illustrated in TABLE 8,* the heart enzymes from different mammalian

* All ratios reported in this paper have been calculated on a basis different from the original work;⁷ they now represent ratios of comparable rates.

species are more closely related to each other than the heart and muscle enzymes of one species. Furthermore, the muscle enzymes of different species are quite similar.

Our analogue studies suggest that there are some differences in the lactic dehydrogenases in mammalian tissues other than in the heart and muscle tissues.

TABLE 8

THE RATIOS OF THE REACTION RATES OF LACTIC DEHYDROGENASES FROM HEART AND MUSCLE OF DIFFERENT MAMMALS*

	APDPN ₁ /TNDPN ₁	
	Heart muscle	Skeletal muscle
Man	0.28	0.72
Rat	0.28	0.96
Mouse	0.48	1.20
Guinea pig	0.36	1.10
Rabbit	0.20	2.00
Beef	0.38	1.20
Lamb	0.28	1.40
Pig	0.28	1.00

* These values were taken from Kaplan *et al.*⁷

TABLE 9

COMPARISON OF THE LACTIC DEHYDROGENASE REACTION RATES OF THE THIONICOTINAMIDE AND ACETYLPIRIDINE ANALOGUES OF DIPHOSPHOPYRIDINE NUCLEOTIDE IN DIFFERENT HUMAN TISSUES

	APDPN(1)/TNDPN(1)			
	Heart	Liver*	Kidney	Muscle
1	0.28	1.00	0.22	0.69
2	0.27	0.80	0.21	0.61
3	0.24	0.98	0.28	0.64
4	0.32	1.10	0.25	0.71

* When using crude extracts of liver, erroneous results may be obtained with the APDPN. This is because the analogue reacts readily with the ethanol and the liver alcohol dehydrogenase. It is possible that ethanol is present in some lactate samples, and this may interfere when a high level of lactate is employed. We have overcome this interference by including hydroxylamine when APDPN is used with liver extracts. Hydroxylamine is a potent inhibitor of liver alcohol dehydrogenase.¹⁷

These differences are illustrated in TABLE 9, which compares some human tissues. The liver enzyme is more closely related to the muscle enzyme than to the heart enzyme. The kidney enzyme, however, seems to be more closely related to the heart lactic dehydrogenase. With some of our newer coenzyme analogues it has been possible to distinguish between the heart and kidney lactic dehydrogenases.

A preliminary survey, based on the analogue reactions, suggests that there may be two main types of lactic dehydrogenases in human tissues (TABLE 10). This classification was obtained from results primarily with the thionicotin-

amide-*DPN and 3-acetylpyridine-*DPN reactions. Lung seems to be intermediate between the two groups. Although the tissues appear to fall into two main categories, there may be more subtle differences among the members of each group. Our present working hypothesis is that the two main forms represent different molecular configurations that are modified in the individual tissues.⁷ An understanding of the genetic factors controlling the formation of the lactic dehydrogenases in various tissues may be of considerable importance for the solution of some of the fundamental problems of differentiation.

It may be worthwhile to compare our results in relation to those obtained by electrophoretic procedures in which multiple bands of lactic dehydrogenases have been reported to occur in extracts of mammalian tissues.⁹⁻¹¹ Our methods with the coenzyme analogues would not discriminate among the bands and would represent the average activities of the bands in each tissue. However, it should be pointed out that most of the activity in each tissue resides in one or two main electrophoretic fractions. Markert and Appella report, elsewhere in these pages, that activity of two significant electrophoretic components of

TABLE 10
TYPES OF HUMAN LACTIC DEHYDROGENASES

I	II
Skeletal muscle Liver Spleen Intestine	Heart Kidney Pancreas Stomach Erythrocytes
Lung	

beef heart are identical in their physical characteristics as well as in their reaction rates with the dinucleotide analogues. It therefore appears quite likely that in our experiments, even in crude extracts, we are measuring the main catalytic characteristic that is significant in the tissue.

We have also found that the lactic dehydrogenases from lobster tissues other than the heart possess the same relatively low affinity that the heart enzyme has for lactate when DPN is the coenzyme, and a much greater affinity for the substrate when the acetylpyridine DPN is the hydrogen acceptor. This is shown in TABLE 11. The heart enzyme from the lobster seems to be somewhat different from the thoracic muscle enzyme, and the thoracic muscle enzyme appears to differ slightly in some characteristics from the claw muscle. Although there are some differences in the individual tissue enzymes of the lobster, all the tissue enzymes seem to have common characteristics. The other crustaceans listed in the table also contain lactic dehydrogenases with properties similar to those of the lobster. On the other hand, *Limulus* (horseshoe crab), which is a member of the *Arachnids*, appears to contain lactic dehydrogenases considerably different from those of the crustaceans. The enzymes of the *Limulus* react more favorably with DPN than does the lobster system, as shown by the low acetylpyridine ratios. All the lactic dehydrogenases of the *Limulus* that have been studied show a relatively low affinity for lactate when the acetylpyridine-

*DPN is used as coenzyme. The *Limulus* hearts can also be characterized by their inactivity with the pyridine-3-aldehyde and thionicotinamide analogues; the crustacean enzymes all appear to react somewhat with these analogues. It is noteworthy that scorpions, spiders, and tarantulas, which are in the same subphylum as *Limulus*, have lactic dehydrogenases with properties very similar

TABLE 11
LACTIC DEHYDROGENASES OF THE PHYLUM ARTHROPODA*

Subphylum	DPNH(2) DPNH(1)	DeDPNH(2) DPNH(2)	DPN(2) DPN(1)	APDPN(1) DPN(1)	Detectable TNDPN	Activity Py3AlDPN
Mandibulata:						
hermit crab body muscle	1.3	0.9	9.4	6	+	+
fiddler crab claw muscle	1.8	0.2	>10	>40†	+	+
green crab body and claw muscle	1.7	0.3	6.0		+	+
edible crab body muscle	2.2	0.3	>10		+	+
crayfish body muscle	2.8	0.2	>10		+	+
lobster heart muscle	3.0	0.17	3.9	35	+	+
body muscle	1.2	0.7	9.0	17	+	+
claw muscle	2.0	0.3	4.0	40	+	+
Chelicerata:						
limulus heart muscle	0.36	2.7	1.1	0.30	—	—
body muscle	0.59	1.8	1.5	0.08	—	—
liver	0.29	8.3	1.4	0.70	—	—
tarantula muscle	0.5	1.1	1.3	0.12	—	—
scorpion muscle	0.4	3.5	1.6	0.16	—	—
wolf spider muscle	0.35	1.8	1.5	0.06	—	trace

* Adapted from Kaplan *et al.*⁷

† The activity with low concentrations of lactate and DPN were most slow, making it impossible to obtain significantly accurate ratios.

TABLE 12
LACTIC DEHYDROGENASES OF SOME ANNELIDS

	DeDPN(2) DeDPN(1)	APDPN(1) DPN(1)	DPNH(2) DPNH(1)	DPNH(1) DeDPNH(1)	DeDPNH(2) DeDPNH(1)	Py3AlDPN(2) DPN(2)
Earthworm (I)	5.3	7.5	1.2	2.4	1.9	0.66
Earthworm (II)	5.0	6.1	1.4	2.5	2.1	0.90
Leech (medical)	5.2	3.6	1.1	2.2	1.7	0.77
Leech (nonmedical)	4.8	3.5	1.1	1.9	1.6	0.65
<i>Nereis</i>	2.2	0.65	1.0	1.7	1.0	0*

* No reaction with Py3AlDPN.

to those of the *Limulus*. Although lactic dehydrogenases from the different tissues of the *Limulus* appear to have some similar properties, there are characteristics that show that the enzymes from the individual tissues are heterogeneous. Such evidence may further indicate that the lactic dehydrogenases of one species may possess common properties which are modified in different tissues.

TABLE 12 compares the properties from a number of annelid species. It is evident that the two different species of earthworm are closely related, and the two species of leech also show enzymes with the same general characteristics.

It is interesting that the enzymes from the two leeches are more closely related to each other than to the enzymes from the two earthworms; this is also true for the earthworms. On the other hand, the lactic dehydrogenase obtained from the muscle of the *Nereis* appears to be significantly different from the earthworm and leech enzymes.

We have previously reported that the flat fish possess lactic dehydrogenases that are quite strikingly different from those of other fish (TABLE 13). Various flat fish, such as sole, flounder, and halibut, apparently have closely related lactic dehydrogenases. Although the heart and skeletal muscle enzymes of the flat fish appear to be somewhat different, they still possess the same general characteristics of reacting rapidly with the acetylpyridine analogue. This again would indicate common characteristics for a given species. It is interesting that the malic and liver alcohol dehydrogenases of the flat fish are considerably different from those of other vertebrates.

It has been reported that the flat fish group originated in the Eocene period, which is about 50 million years later than when most teleost groups existed.

TABLE 13
APDPN(1)/TNDPN(1) LACTIC DEHYDROGENASE RATIOS IN FISH HEART

Man	0.28	Cod	1.52
Sea bass	0.36	Silver hake	0.70
Butterfish	0.32	Perch	4.2
Scup	0.24	Puffer	3.0
Sea robin	0.76	Flounder	11.2
Toadfish	0.56	Sole	12.0
Dogfish	0.48	Halibut	11.6
Shark	1.36		

As indicated in the table, the perch heart lactic dehydrogenase, when compared with the other fish, shows characteristics somewhat similar to the flat fish enzymes. It has been suggested that the perch group might be the ancestor of the flat fish. Our data support such a view and illustrate the usefulness of this type of enzyme comparison in evaluating the relationships of different organisms.

In examining some of the fish, we observed that the dark and light muscles of certain species contained lactic dehydrogenases considerably different in their reaction to the coenzymes.⁷ These differences are illustrated in TABLE 14, which shows that, in the trout and mackerel, the enzyme from the dark muscle is quite distinct from that of the light muscle and similar to that found in the heart. In the herring, scup, butterfish, and dogfish, there is also a closer relationship between the heart and dark muscle dehydrogenases than between the dehydrogenases from the dark and light muscles. These results suggest that in these species the heart and dark muscles may have a common origin. In other fish, such as the sea robin, a similarity in properties of the enzymes from the dark and light muscles has been found. These enzymes can easily be distinguished from the heart lactic dehydrogenase (TABLE 14). As indicated in the table, the dark and light muscle enzyme activities of the salamander are almost identical and quite different from those of the heart. According to our

methods of assay, the lactic dehydrogenases from the dark and light muscles of the chicken are also identical.

TABLE 15 compares the reaction rates of the lactic dehydrogenases from the leg and abdomen muscles of the rat. The unusually good agreement of the values obtained indicates that the enzymes in these two skeletal muscles are

TABLE 14

COMPARISON OF RATIOS OF REACTION RATES FOR LACTIC DEHYDROGENASES FROM HEART WITH RATES FOR DARK AND LIGHT MUSCLE ENZYMES OF DIFFERENT SPECIES

	Ratios for LDH from muscle in											
	Mackerel			Trout			Sea robin			Salamander		
	Heart	Dark	Light	Heart	Dark	Light	Heart	Dark	Light	Heart	Dark	Light
$\frac{\text{DPNH}(3)}{\text{DPNH}(1)}$	0.9	0.9	3.2	0.9	0.9	2.5	1.1	1.7	1.4	0.9	1.3	1.1
$\frac{\text{DPN}(3)}{\text{DPN}(1)}$	1.5	1.5	2.7	1.6	1.9	3.2	1.3	2.9	3.2	1.5	2.1	1.9
$\frac{\text{DeDPN}(3)}{\text{DeDPN}(1)}$	1.3	2.0	3.9	2.0	1.7	3.9	2.9	4.6	4.1	2.8	3.2	3.1
$\frac{\text{APDPN}(1)}{\text{DPN}(1)}$	0.1	0.1	0.7	0.2	0.3	1.7	0.2	1.0	1.0	0.2	0.4	0.4
$\frac{\text{APDPN}(1)}{\text{Py3AlDPN}(1)}$	0.6	0.5	2.7	0.8	1.2	11.8	1.5	3.6	2.4	0.8	2.3	2.2
$\frac{\text{APDPN}(1)}{\text{TNDPN}(1)}$	0.3	0.2	4.6	0.6	0.9	9.6	0.8	4.1	3.5	0.6	1.6	1.6

TABLE 15

COMPARISON OF SEVERAL RAT MUSCLE LACTIC DEHYDROGENASES

	$\frac{\text{DPN}(3)}{\text{Deamino DPN}(1)}$	$\frac{\text{TNDPN}(3)}{\text{TNDPN}(1)}$	$\frac{\text{DPN}(2)}{\text{DPN}(1)}$
Skeletal leg muscle	4.9	4.0	2.0
Skeletal abdominal muscle	5.0	3.9	2.2
Diaphragm	2.6	2.1	1.6

identical. The diaphragm, however, appears to contain an enzyme somewhat different from that contained in the other two muscles.

Markert and Møller¹¹ and Flexner *et al.*¹² have reported changes by electrophoretic methods of the lactic dehydrogenases in individual tissues during development. By means of the analogues, we have been able to demonstrate that newborn rat heart contains lactic dehydrogenases somewhat different from those found in the adult organ (TABLE 16). This difference has been confirmed by immunological studies. The good agreement of the values obtained from

the individual animals suggests the validity of this finding. It is of interest that we have detected by our methods no difference between the newborn and adult skeletal muscle enzymes. Although Flexner *et al.*¹² have reported a difference between the newborn and adult guinea pig liver lactic dehydrogenases, we have been unable to show any significant difference between the newborn and adult rat liver enzymes. The catalytic technique appears to be a potent tool for detection of changes in the nature of the dehydrogenases during ontogeny and growth.

In our initial studies we were looking for rather large differences in ratios to detect heterogeneity. More recently, we have been checking for variations in more closely related species. A comparison of the activities of the crystalline lactic dehydrogenases from beef heart and skeletal muscle with those of the

TABLE 16
ADULT AND NEWBORN RAT HEART LACTIC DEHYDROGENASES

	$\frac{\text{APDPNH}(2)}{\text{APDPNH}(1)}$	$\frac{\text{EPKDPNH}(2)}{\text{NeEtDPNH}(2)}$
Adult		
1	0.94	—
2	0.89	1.17
3	1.05	1.10
4	0.80	1.36
5	0.95	1.10
6	0.92	1.42
Newborn*		
1	1.25	—
2	1.40	0.48
3	1.50	0.43
4	1.32	0.78
5	1.22	0.73
6	1.36	0.50

* All were less than 24 hours old.

purified enzymes from chicken heart and muscle indicates that there are significant differences between the chicken and beef systems; these are shown in TABLE 17. The chicken muscle enzyme reacts at a considerably faster rate with the acetylpyridine-*DPN relative to the natural coenzyme than does the corresponding beef catalyst. The differences between the two heart enzymes are reproducible. Although the two heart proteins have many common characteristics, they are certainly not identical. It is of interest that Robert D. Cahn and Lawrence Levine of our laboratory have found that an antibody produced against the chicken heart lactic dehydrogenase will cross-react with the beef heart enzyme. The antibody reaction with the beef heart protein is much weaker than with the homologous chicken heart dehydrogenase. Nevertheless, the beef heart enzyme shows a better cross reaction with the antibody than do either the beef or chicken skeletal muscle enzymes. In general, we have been able to obtain good agreement between immunochemical and coenzyme analogue methods in showing the relationships of the dehydrogenases (see also Nisselbaum and Bodansky^{13,14}). We have been able to predict, with some ac-

curacy, the cross reactivity of various enzymes to a given antibody by an examination of the catalytic data obtained with the analogues.

The ability to distinguish chicken and beef enzymes has prompted us to investigate carefully possible variations among the dehydrogenases of different mammalian species; this type of study is now in progress.

TABLE 17
COMPARISON OF BEEF AND CHICKEN LACTIC DEHYDROGENASES

	Heart		Skeletal muscle	
	Beef	Chicken	Beef	Chicken
$\frac{\text{DPN}(2)}{\text{DPN}(1)}$	1.12	1.22	2.18	2.62
$\frac{\text{TNDPN}(1)}{\text{DPN}(1)}$	0.36	0.58	0.22	0.24
$\frac{\text{APDPN}(1)}{\text{DPN}(1)}$	0.06	0.15	0.23	1.04
$\frac{\text{APDPN}(3)}{\text{APDPN}(1)}$	0.67	0.28	1.02	0.96

TABLE 18
COMPARISON OF RATIOS OF REACTION RATES OF REDUCED ANALOGUES OF DPN WITH CRYSTALLINE BEEF HEART AND BEEF SKELETAL MUSCLE LACTIC DEHYDROGENASES

	Beef heart	Beef muscle
$\frac{\text{Deamino-DPNH}}{\text{Acetylpyridine-*DPNH}}$	2.15	1.03
$\frac{\text{Acetylpyridine-*DPNH}}{\text{Ethyl pyridyl ketone-*DPNH}}$	1.45	8.90
$\frac{\text{Ethylpyridyl ketone-*DPNH}}{6-(2\text{-hydroxyethylamino})\text{purine-DPNH}}$	1.5	0.29

Rates were compared using 4 times as much ethyl pyridylketone-*DPNH, and 6-(2-hydroxyethylamino)purine-DPNH, 10 times as much acetylpyridine-*DPNH as deamino-DPNH.

It is also possible to show differences by means of the reduced coenzyme analogues. This is demonstrated in TABLE 18 where the activities of crystalline beef heart and beef muscle lactic dehydrogenases are compared. The striking differences in catalytic properties are also reflected by great differences in physical properties. Ludwig Brand, Robert H. McKay and Johannes Everse have studied in great detail a number of characteristics of the two enzymes. FIGURE 3 shows the large difference in heat stability of the two crystalline proteins. Other properties, such as sensitivity to urea denaturation and hydrogen ion,

are also extremely different. Preliminary studies with the Stein and Moore automatic amino acid analyzer showed little difference in the amino acid composition of the two lactic dehydrogenases. We emphasize again that when

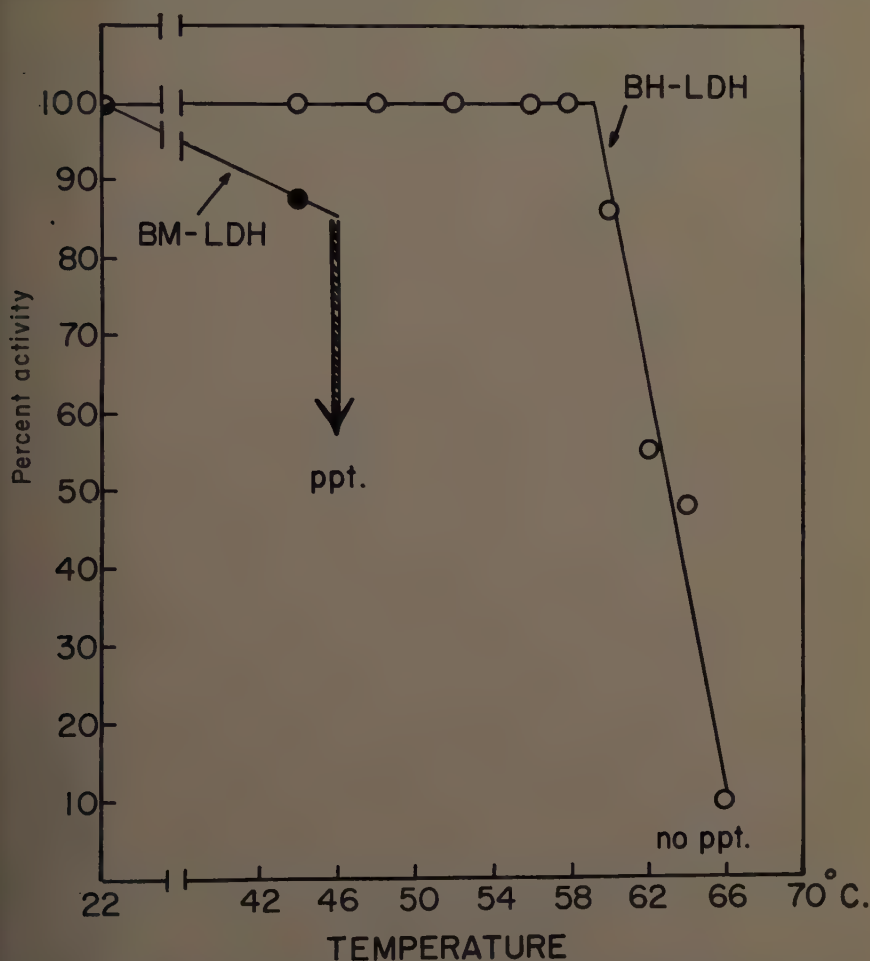


FIGURE 3. Effect of temperature on the reaction rates of crystalline beef heart and beef skeletal muscle lactic dehydrogenases. The reactions were carried out at 30° C. after treatment for 15 min. at the indicated temperature.

differences exist in the catalytic activities of two enzymes promoting the same function, differences in the physical properties of the proteins should also be observable.

Malic Dehydrogenases

During the past two years, we have made an extensive study with the coenzyme analogues of the malic dehydrogenases in different organisms. This

enzyme is of considerable advantage in studies on evolution and classification because of its almost universal distribution. Lactic dehydrogenase, on the other hand, is absent in many plants, invertebrates, and microorganisms.

We have found by the reaction rates with the analogues that the soluble and mitochondrial enzymes are different. This difference has also been reported by others using different criteria.^{15,16} A comparison of the soluble and mitochondrial malic dehydrogenases from several tissues of the rabbit is given in TABLE 19. The soluble fraction represents a high speed (100,000 g for 60 min.) centrifugate. The mitochondrial values were obtained on extracts of mitochondria after sonic oscillation and centrifugation at 100,000 g. Placing the soluble fraction in a sonic oscillator does not alter the catalytic characteristics of the soluble malic dehydrogenase. It is evident from the data in TABLE 19 that the soluble enzymes of different tissues are more closely related to each other than the mitochondrial and soluble enzymes of one tissue. This is also true of the various mitochondrial enzymes. The heart enzyme seems to be

TABLE 19
MALIC DEHYDROGENASES OF SOLUBLE AND MITOCHONDRIAL FRACTIONS OF RABBIT TISSUES

	DPNH(1) DeDPNH(1)		TNDPN(3) TNDPN(1)		EKDPN(1) TNDPN(1)		APDPNH(3) DeDPNH(1)		APDPNH(3) DPN(3)		TNDPN(3) Py3AIDPN(3)	
	Mt Sol		Mt Sol		Mt Sol		Mt Sol		Mt Sol		Mt Sol	
	Mt	Sol	Mt	Sol	Mt	Sol	Mt	Sol	Mt	Sol	Mt	Sol
Liver	2.4	1.4	3.3	2.2	3.6	0.92	1.3	0.36	1.4	0.89	1.7	1.7
Brain	4.2	1.4	2.5	1.7	1.7	0.82	1.5	0.90	1.2	0.52	2.3	2.0
Kidney	4.7	1.8	3.4	1.9	2.9	0.90	1.7	0.44	1.2	0.76	1.9	2.0
Muscle	4.7	1.6	5.2	1.7	4.3	0.92	1.7	0.54	1.5	0.95	1.8	2.1
Heart	3.7	2.2	3.8	1.9	4.2	2.1	3.0	1.8	4.7	1.1	1.7	2.0

somewhat anomalous when compared to the findings of other rabbit tissues. This anomaly, however, has also been found in rat heart, where both the mitochondrial and soluble malic dehydrogenases appear to be distinct from the corresponding enzymes in kidney, muscle, and liver.

Our studies to date suggest there is not the striking difference in the individual tissue malic dehydrogenases of mammals as has been found with the lactic dehydrogenases. The evolutionary changes that have been observed with the mammalian and fish lactic dehydrogenases are not so apparent with the malic dehydrogenases. Some differences have been detected among a number of teleost fish and mammals, however. More dramatic differences have been observed with invertebrates. This is illustrated in TABLE 20, which compares the soluble malic dehydrogenases of the rabbit with those of the clam, octopus, and snail. The results indicate that each animal has a distinct malic dehydrogenase characterized by its reaction with some of the analogues. The malic dehydrogenases in all the clam tissues can be distinguished by their low affinity for ethyl pyridine ketone-*DPN, as well as by their slow reactions with deamino DPN. The clam enzymes appear to be considerably different from the snail malic dehydrogenases. In the octopus, the enzyme is characterized by a marked substrate inhibition when acetylpyridine-*DPN is the coenzyme. Al-

though all the tissues of the octopus show this substrate inhibition, the individual tissues show a significant difference in the ratios of the reaction rates obtained with thionicotinamide-*DPN and deamino DPN. This would suggest that there is a characteristic species, malic dehydrogenase, which is modified in the different tissues. From our preliminary survey it is our impression that there has been more differentiation of malic dehydrogenases in the tissues of invertebrates than in vertebrates.

The malic dehydrogenases in bacteria show considerable diversification. We have found in general that closely related species possess enzymes with properties which are quite similar. These relationships are illustrated in TABLE 21. It is of interest that members of the *aerogenes-coli* group have malic de-

TABLE 20
MALIC DEHYDROGENASES OF SOME ANIMALS*

	$\frac{\text{APDPN}(3)}{\text{APDPN}(1)}$	$\frac{\text{TNDPN}(3)}{\text{DeDPN}(1)}$	$\frac{\text{EPDPN}(3)}{\text{EPDPN}(1)}$	$\frac{\text{DeDPN}(3)}{\text{DeDPN}(1)}$
Snail				
Kidney	0.43	11.0	2.3	4.3
Liver	0.43	12.0	2.9	3.3
Muscle	0.39	12.3	2.3	3.4
Clam				
Siphon muscle	0.76	>80	16.4	20
Liver	—†	>80	16.0	>20
Foot muscle	0.50	55	12.0	9
Octopus				
Liver	0.10	>20	2.5	>20
Brachial hearts	0.10	3.3	2.4	2.0
Ovary	0.11	3.0	1.9	0.79
Rabbit				
Muscle	0.40	2.0	3.1	3.0
Liver	0.37	3.0	3.6	3.5
Kidney	0.38	2.7	4.1	3.4
Heart	0.41	3.4	3.2	3.1

* The analyses were done on the 100,000 g supernatant of the tissue homogenate.

† Value not reported because of inaccuracy due to interfering reactions.

hydrogenases with common characteristics and that they are quite different from those of the other bacteria listed in the table.

In contrast to the *aerogenes-coli* group, the *Bacillus* group as a whole shows remarkable differences with respect to their malic dehydrogenases. We have studied to date approximately 50 members of this group, and we have been greatly impressed by the extent of the diversification. TABLE 22 gives the data on some of the organisms that we have studied. The data with the analogues clearly indicate a difference between *B. megatherium*, *B. cereus*, *B. macerans*, and *B. subtilis*. As indicated, the malic dehydrogenases of *B. mycoides*, which is a closely related variant of *cereus*, has characteristics almost identical with the *cereus* enzyme. The *megatherium-cereus* organism, a strain supplied to us by Jackson Foster, has been considered an intermediate between the two types of *Bacillus*. Our results with the malic dehydrogenases indicate that the organism is very closely related to *cereus*, and not to *megatherium*.

Julius Marmur of our laboratory has been making a detailed study of the base composition in the DNA of the *Bacillus* group. In contrast to the *aerogenes-coli* group, he has found considerable differences in the composition of the DNA in the various *Bacillus* members. It is of interest, in this connection, that the DNA of the *megatherium-cereus* intermediate is almost identical in composition to that of *cereus*. A generally good correlation has been found between our

TABLE 21
BACTERIAL MALIC DEHYDROGENASES

	DPNH(1) DPNH(2)	DPNH(1) DeDPNH(1)	TNDPN(3) Py3AlDPN(3)	APDPN(1) DPN(1)	DPN(3) TNDPN(3)	APDPN(3) APDPN(1)
<i>Coli aerogenes</i> group:						
<i>E. coli</i>	2.4	3.6	1.6	4.8	3.6	0.45
<i>Aero. aerogenes</i>	2.4	2.9	1.9	4.8	3.3	0.46
<i>Sh. dysenteriae</i>	3.0	2.9	1.7	4.0	3.7	0.43
<i>Salm. typhimurium</i>	2.4	3.0	1.6	4.5	3.9	0.42
Other bacteria:						
<i>B. subtilis</i>	4.0	0.91	1.2	2.0	0.47	0.15
<i>Rhizobium</i> (Str. 505)	3.0	0.8	7.0	9.9	1.1	0.90
<i>Pr. pentosaceum</i>	1.3	2.8	5.7	2.7	1.9	0.43
<i>Ps. saccharophila</i>	1.0	1.8	1.3	1.3	2.9	0.37
<i>R. rubrum</i>	2.5	3.1	3.3	7.3	1.2	0.45
<i>Achromo. fischeri</i>	2.9	2.3	1.7	6.2	1.8	0.43
<i>Lacto. arabinosus</i>	1.5	7.4	1.8	5.7	3.1	0.38

TABLE 22
BACILLUS MALIC DEHYDROGENASES

	APDPNH(1) APDPNH(3)	DPN(2) EPKDPN(2)	DPNH(3) APDPNH(3)	BPK- *DPN†	TNDPN(3) TNDPN(1)
<i>Cereus</i>	1.4	>100	1.6	0	1.6
<i>Cereus v. mycoides</i>	1.4	>100	2.0	0	1.3
" <i>Cereus-megatherium</i> "	1.3	>100	1.8	0	1.6
<i>Megatherium</i>	5.0	1.1	19.8	0	4.4
<i>Macerans</i> No. 1	4.8	0.84	27.0	+	2.1
<i>Macerans</i> No. 2	5.5	0.70	21.2	+	1.8
<i>Subtilis</i> No. 9789	0.68	14.2	0.56	trace	0.58
<i>Subtilis</i> No. 168	0.81	16.0	0.75	trace	0.57

† Butyryl pyridyl ketone-*DPN.

results with the malic dehydrogenase and Marmur's DNA studies. Further details of these investigations will be published elsewhere.

We are at present continuing our survey of the bacterial enzymes. It is our opinion that characterization of the malic dehydrogenases may prove to be a useful and accurate adjunct for classifying microorganisms.

TABLE 23 shows that the malic dehydrogenases in a number of higher plants are closely related. The enzymes from the two unicellular organisms, *Nitella* and *Chlorella*, have characteristics that indicate their relationships and are somewhat distinct from the higher plant catalysts.

As indicated by the results with yeast and *Neurospora*, the fungal malic de-

hydrogenases are different from those of the green plants. The slime mold and *Euglena* dehydrogenases appear not to be related to those of the plants.

Our findings suggest that there have been marked changes in malic dehydrogenase during evolution. Although the results of our studies are not conclusive, it is our view from an analysis of the data available thus far that the malic dehydrogenases represent the diversification of a single evolutionary change. On the other hand, we believe that the lactic dehydrogenases in vertebrates, invertebrates, and bacteria may have arisen independently from each other as separate evolutionary events.

The results of our studies strongly suggest that there has been considerable evolutionary change in the lactic and malic dehydrogenases. It appears that enzyme change is not without significance, but is of importance in the survival and perpetuation of new species. This is indicated by the fact that although

TABLE 23
COMPARISON OF SOME HIGHER PLANT MALIC DEHYDROGENASES WITH OTHER ORGANISMS

	DeDPNH(2) DeDPNH(1)	DPN(2) Py ₃ AlDPN(2)	APDPN(1) DPN(1)	TNDPN(3) TNDPN(1)
Corn	1.7	17	8.0	2.9
Peas	1.6	15	9.2	3.0
Sea grass	—*	15	11.5	3.7
Wheat germ	1.9	12	11.0	3.1
Ryegrass seed	1.7	8	9.6	2.5
<i>Nitella</i>	1.5	>50	5.4	2.5
<i>Chlorella</i>	1.6	>100	7.4	1.8
Yeast	1.0	5.3	22	15
<i>Neurospora</i>	1.0	5.5	13	23
<i>Euglena</i>	0.8	7.0	3.2	3.2
Slime mold	0.82	1.0	0.48	1.0

* The sea grass malic dehydrogenase does not show a significant reaction with deamino DPNH.

there is a characteristic mammalian lactic dehydrogenase in muscle, the enzyme is diversified to some extent in the individual members of this group. It is also our belief that the lactic dehydrogenase in vertebrates played a significant role in the evolution of this phylum. Further discussion of the evolutionary significance of the dehydrogenases will be presented elsewhere.

It is evident from our studies that the catalytic identification of enzymes is of considerable value in phylogenetic and taxonomic studies. We believe that our approach can also be applied to other than pyridine nucleotide-linked enzymes. Comparative enzyme structure also appears to be of importance in classification studies as well as in evaluations of changes associated with evolution. It is our hope that studies on some of the crystalline dehydrogenases may help us ascertain which part of the enzyme molecule undergoes change during evolution.

Acknowledgment

We thank Natalie Grimes and Clarence Baker for their assistance in part of the work described in this article.

References

1. VALLEE, B. 1960. *In* The Enzymes. Boyer, Lardy, and Myrback, Eds. **3b**: 225. Academic Press. New York, N.Y.
2. DENNIS, D. & N. O. KAPLAN. 1960. J. Biol. Chem. **235**: 810.
3. LAMBORG, M. & N. O. KAPLAN. 1960. Biochim. et Biophys. Acta. **38**: 272.
4. HORWITZ, S., M. LISS & N. O. KAPLAN. 1961. Bacteriol. Proc.
5. KAPLAN, N. O., M. M. CIOTTI & F. E. STOLZENBACH. 1956. J. Biol. Chem. **221**: 833.
6. VAN EYS, J., A. SAN PIETRO & N. O. KAPLAN. 1958. Science. **127**: 1443.
7. KAPLAN, N. O., M. M. CIOTTI, M. HAMOLSKY & R. E. BIEBER. 1960. Science. **131**: 392.
8. KAPLAN, N. O. 1959. Ciba Foundation Study Group No. 2. Steric Course of Microbiological Reactions. Wolstenholme and O'Connor, Eds. 37. Churchill, London, England.
9. VESELL, E. A. & A. G. BEARN. 1958. J. Clin. Invest. **37**: 672.
10. WIELAND, T. & G. PFLEIDERER. 1957. Biochem. Z. **329**: 112.
11. MARKERT, C. L. & F. MØLLER. 1959. Proc. Natl. Acad. Sci. U.S. **45**: 753.
12. FLEXNER, L. B., J. B. FLEXNER, R. B. ROBERTS & G. DE LA HABA. 1960. Developmental Biology. **2**: 313.
13. NISSELBAUM, J. S. & O. BODANSKY. 1959. J. Biol. Chem. **234**: 3276.
14. NISSELBAUM, J. S. & O. BODANSKY. 1961. J. Biol. Chem. **236**: 323.
15. DELBRUCK, A., H. SCHIMASSEK, K. BARTSH & T. BUCHER. 1959. Biochem. Z. **331**: 297.
16. THORNE, C. J. R. 1960. Biochim. et Biophys. Acta. **42**: 175.
17. KAPLAN, N. O. & M. M. CIOTTI. 1953. J. Biol. Chem. **201**: 785; KAPLAN, N. O., M. M. CIOTTI & F. E. STOLZENBACH. 1954. J. Biol. Chem. **211**: 419.

Discussion of the Paper

A. SAMUELS (*Department of Pathology, Dartmouth Medical School, Hanover, N.H.*): Did the substrate lactate protect the lactic dehydrogenase against antibody inhibition?

KAPLAN: No.

SAMUELS: This has also been pointed out by B. Cinader,* in his review of antibodies against enzymes, who set up a table postulating the combinations of substrate and coenzymes that should be needed to protect the enzyme against antibody inhibition, given a mechanism of steric inhibition. Following this lead, the protection of the crystalline enzyme Creatine-ATP transphosphorylase against its antibody was studied, and it was found that all substrates (not only two) were necessary for protection, which indicated that not only are steric factors necessary for enzyme inhibition by antibody and protection by substrate against inhibition, but that the *conformation* of the enzyme is changed during the combinations,† as further shown by studies of the rotation change during antigen-antibody formation,‡ and by the rotatory dispersion change of creatine kinase *only* on activation by both Mg^{++} .†

Regarding the change in kinetic and inhibitory characteristics of lactic dehydrogenase from skeletal muscle during development, may I report that we have done similar work on 5'-adenylic acid deaminase in developing chick-embryo skeletal muscle. This enzyme appears in the embryo muscle at 7 days of embryonic age, and increases in content with age. The apparent K_m of the enzyme studied, on preparations that have undergone various degrees of purifi-

* Ann. Rev. Microbiol. 1957. **11**: 371.

† SAMUELS, A. 1961. Biophys. J. **1**(6): 437. Biophys. Soc. 1961. 5-TB-9.

‡ CAMPBELL, D. H. & K. ISHIZAKA. 1959. **83**: 318.

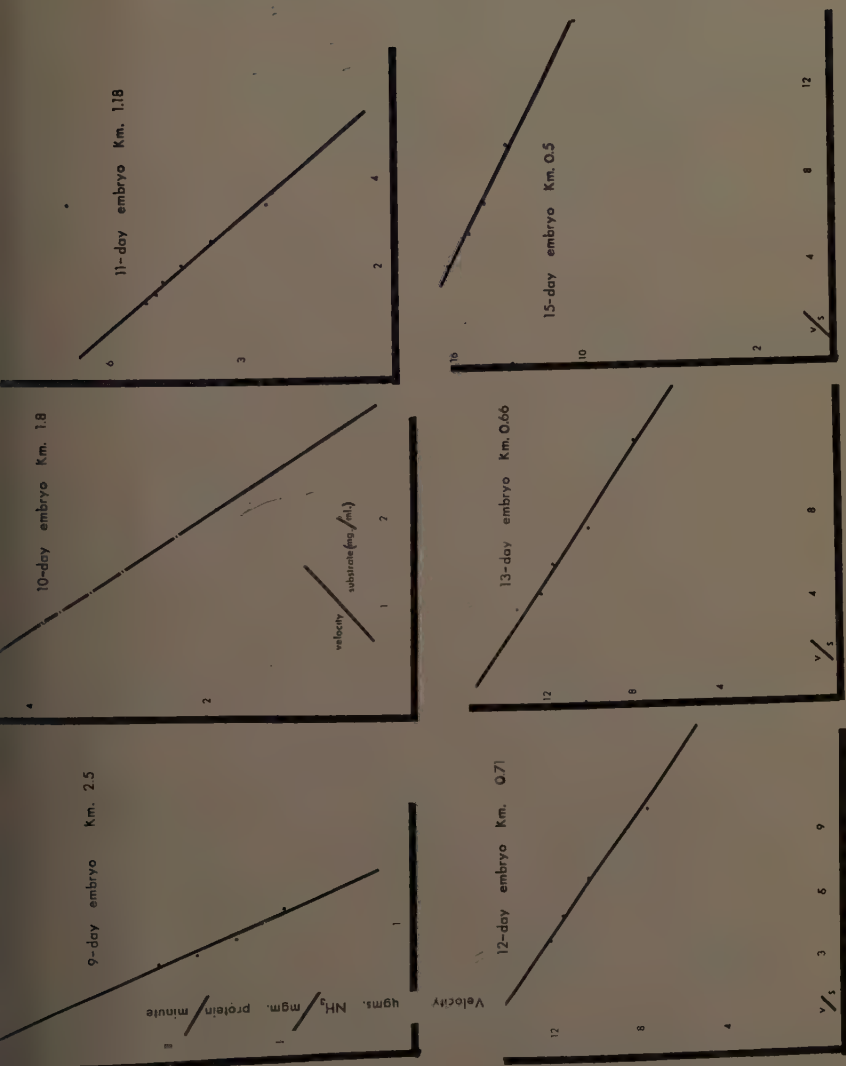


FIGURE 1. Graphic determination of the Michaelis-Menten constants. The K_m values decreased from $7.2 \times 10^{-3} M$ at 9 days embryonic age to $1.4 \times 10^{-3} M$ at 15 days. The NH_3 liberation was determined using KCl-succinate (pH 5.9) buffer, 5'-adenylic acid over a concentration range of 2.1×10^{-3} to $12.9 \times 10^{-3} M$.

cation, decreases fivefold between 9 and 14 days from $7.2 M$ to $1.4 M \times 10^{-3}$, after which time it maintains the K_m value of the adult enzyme ($1.4 \times 10^{-3} M$), although the concentration continues to increase as the animal grows. This is shown in FIGURE 1* (this work was done in conjunction with work carried on in the Laboratory of Chemical Embryology, Denver, Colo., by H. Herrmann).

* Reported in *The Physiologist*, 1957. 1: 24.

MULTIPLE PEROXIDASES IN CORN*

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The physiological role of peroxidase in the plant is not clear, but the increased peroxidase activity of dwarf plants (Kamerbeek, 1956) and slowly growing tissues (Galston and Dalberg, 1954; Konishi, 1956) suggests that peroxidase may be a growth-inhibiting factor in the plant. Peroxidase *in vitro* mediates the oxidation of the growth hormone indoleacetic acid (IAA) as discussed by Galston *et al.*, 1953 and by Ray, 1960; the formation of lignin or ligninlike substances from coniferyl alcohol or eugenol (Higuchi, 1957; Siegel, 1956); and the oxidation of oxalate, oxalacetate (Kenten and Mann, 1953), TPNH, DPNH (Azakawa and Conn, 1958), dihydroxyfumarate (Chance, 1952), and many other compounds (Burris, 1960). The oxidation *in vivo* of cytochrome C by peroxidase in yeast has also been shown (Chance, 1954). Thus peroxidase could limit growth by limiting the amount of IAA present, by the formation of a rigid lignin matrix in cell walls, or by affecting respiration in the plant.

Since the occurrence of multiple peroxidases in plants has been shown by zone electrophoresis (Galston and McCune, 1961; Garay and Faragó, 1959; Jermyn and Thomas, 1954; Markert and Møller, 1959), by ion exchange chromatography (Paul, 1958); and by various fractionation procedures (Hosoya, 1960a; Kondo and Morita, 1952), the physiological role of peroxidase and the significance of multiple forms of enzymes have become closely related problems. To obtain greater understanding of these problems the substrate specificities of the individual peroxidases of the corn leaf sheath and the changes in the peroxidase complement of this tissue occurring with growth, dwarfism, and the application of gibberellic acid were studied.

Materials and Methods

Seed of *Zea mays* L. was given by Bernard O. Phinney of the University of California, Los Angeles, Calif. Segregating dwarf-1 (d_1), dwarf-3 (d_3), dwarf-5 (d_5), and dwarf-8 (d_8 or dominant) stocks were used. The plants were grown under conditions previously described (McCune and Galston, 1959). Preparation of the material and starch block electrophoresis were carried out by previously described procedures (Galston and McCune, 1961).

Peroxidase activity was determined using guaiacol as an hydrogen donor, and the formation of tetraguaiacol was followed at 470 $m\mu$ in a recording spectrophotometer (model 3000 Perkin-Elmer Spectracord) at 30° C. The reaction mixture contained $1.2 \times 10^{-2} M$ guaiacol, $5 \times 10^{-3} M$ H_2O_2 (reagent A.C.S., 30 per cent solution),[‡] and pH 6.1, 0.02 M phosphate buffer. After an initial

* This paper constitutes part of a dissertation presented in partial fulfillment for the degree of Doctor of Philosophy, Yale University, New Haven, Conn.

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[†] Eastman Kodak Co., Rochester, N.Y.

[‡] Allied Chemical Corp., New York, N.Y.

lag period the rate of increase of absorbancy was constant for more than 2 min. and was used as the peroxidase activity of the sample. During the course of the assay less than 4 per cent of the substrate was used.

When pyrogallol was used as an hydrogen donor the increase in absorbancy was measured at 430 $m\mu$.

The oxidation of IAA was determined by the method of Ray (1956). The reaction mixture contained $1.7 \times 10^{-4} M$ IAA* and pH 6.1, 0.02 M phosphate buffer. In some instances $MnCl_2$ ($8.3 \times 10^{-5} M$), 2,4-dichlorophenol (DCP) (8.3×10^{-5}), or H_2O_2 ($6.3 \times 10^{-3} M$) were included in the reaction medium.

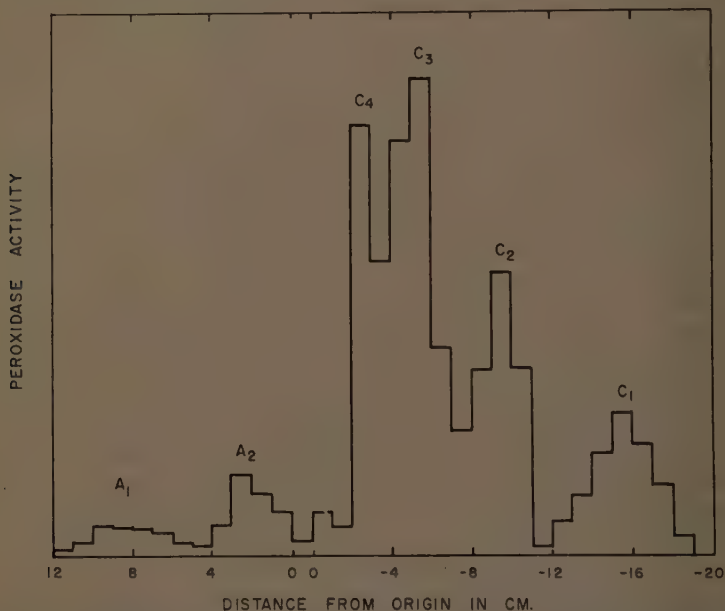


FIGURE 1. Fractionation of corn-leaf sheath peroxidases by starch electrophoresis in phosphate buffer (pH 6.1, 0.02 M) at 2° C. for 12 hours with a field of 6.3 v/cm.

The temperature was 30° C. Quartz cuvettes of 1.0 cm. path length were used, and the absorption spectrum of the reaction mixture from 330 to 230 $m\mu$ was determined at 3 or 5 min. intervals. The reaction mixture without added IAA was the blank. After an initial lag period the increase in absorbancy at 261 $m\mu$ was constant and used as the IAA oxidase activity of the sample.

Protein concentrations were determined spectrophotometrically (Layne, 1957).

Results and Discussion

Six peroxidase active fractions were obtained from corn leaf sheath preparations. A representative fractionation is shown in FIGURE 1. After starch

* Nutritional Biochemicals Corp., Cleveland, Ohio.

electrophoresis in pH 6.1, 0.02 *M* phosphate buffer at 2° C. for 12 hours with a field of 6.3 volts/cm., 2 fractions were located 8 cm. (*A*₁) and 3 cm. (*A*₂) toward the anode from the origin, and 4 fractions were located 16 cm. (*C*₁), 10 cm. (*C*₂), 6 cm. (*C*₃), and 3 cm. (*C*₄) towards the cathode from the origin. The starch segments containing the individual fractions were removed and placed in new starch blocks. On a repeated electrophoresis each fraction migrated as a single component.

Substrate specificities of the cathodic components. The activity of each peroxidase as a function of guaiacol, pyrogallol, or hydrogen peroxide concentration was determined; the results are summarized in TABLE 1. Since the *K*_s values shown were determined from steady-state rates, they may not represent affinities but they do serve as indices for comparing the different peroxidases with respect to a given substrate. The *K*_s values for *C*₂, *C*₃, and *C*₄ were

TABLE 1
SUBSTRATE SPECIFICITY OF THE CATHODIC PEROXIDASES

		Peroxidase fraction			
		<i>C</i> ₄	<i>C</i> ₃	<i>C</i> ₂	<i>C</i> ₁
Guaiacol	<i>K</i> _s × 10 ⁻³ <i>M</i> (H ₂ O ₂ 5 × 10 ⁻³ <i>M</i>)	7.1	7.1	7.7	20
Pyrogallol	<i>K</i> _s × 10 ⁻³ <i>M</i> (H ₂ O ₂ 5 × 10 ⁻³ <i>M</i>)	2.1	4.3	1.3	7.1
H ₂ O ₂	<i>K</i> _s × 10 ⁻⁴ <i>M</i> (Guaiacol 2.4 × 10 ⁻² <i>M</i>)	7.1	11	7.7	4.0
Δ <i>A</i> ₄₇₀ /min.	Guaiacol 1.2 × 10 ⁻² <i>M</i> H ₂ O ₂ 5 × 10 ⁻³ <i>M</i>	10.0	10.0	10.0	10.0
Δ <i>A</i> ₄₃₀ /min.	Pyrogallol 5 × 10 ⁻² <i>M</i> H ₂ O ₂ 5 × 10 ⁻³ <i>M</i>	2.5	1.68	6.25	0.23
Δ <i>A</i> ₂₆₁ /hour	IAA 1.7 × 10 ⁻⁴ <i>M</i>	0	0	0	0
	+ MnCl ₂ 8.3 × 10 ⁻⁵ <i>M</i>	0	0	0	0
	+ H ₂ O ₂ 6.2 × 10 ⁻³ <i>M</i>	0.085	0.042	0.270	0.075
	+ DCP 8.3 × 10 ⁻⁴ <i>M</i>	0.395	0.009	0.081	0.017
	+ DCP & MnCl ₂	2.310	0.220	1.780	0.073

similar for guaiacol but excess guaiacol at 7 × 10⁻² *M* inhibited both *C*₃ and *C*₄. The *K*_s of each peroxidase for pyrogallol was different. *C*₂ and *C*₄ were similar with respect to H₂O₂ and *C*₃ was inhibited by excess H₂O₂ at 7 × 10⁻³ *M*.

In the lower half of TABLE 1 the IAA oxidase activity of each peroxidase with various addenda is compared relative to the activity of the enzyme under the guaiacol assay conditions. The activities of the peroxidases under the pyrogallol assay conditions are also given. Each peroxidase was different in the effects of the addenda upon its IAA oxidase activity and in its IAA oxidase activity relative to the peroxidase activity. None of the peroxidases showed IAA oxidase activity with IAA alone or with the addition of MnCl₂ but all showed activity with the addition of H₂O₂, DCP, or DCP and MnCl₂. Only in *C*₄ was the IAA oxidase activity greater with the addition of DCP than with H₂O₂. The addition of MnCl₂ to the medium containing DCP increased the activity of *C*₁ and *C*₄ 5-fold but increased that of *C*₂ and *C*₃ 20-fold.

Plant peroxidases have broad substrate specificities and many compounds serve as hydrogen donors although a peroxidase specific for the alpha-oxidation

of palmitic, stearic, and myristic acids has been found (Stumpf and Bradbeer, 1959). These substrates may be oxidized peroxidatively if H_2O_2 is present or oxidatively with the addition of phenolic cofactors and manganous or cerous ions (Mudd and Burris, 1959). Hosoya (1960*b* and *c*) found that the multiple peroxidases of the turnip differed in hydrogen donor specificity and in *pH* optimum but were similar in reaction mechanism to each other and to horse radish peroxidase. Thus the peroxidases of the plant may be called multiple enzymes on the basis of their similar reactions *in vitro*, but their broad and different substrate specificities indicate that the peroxidases may function by similar mechanisms but mediate different reactions in the plant such as the formation of lignin, the oxidation of dihydroxy fumarate, or the oxidation of IAA. The different responses to *pH*, substrate concentration, or the presence of cofactors may also indicate that these enzymes mediate the same reaction in the plant but at different *pH* values, concentrations of substrate, cofactor, or inhibitor.

TABLE 2
CHANGES IN THE PEROXIDASES OF THE LEAF SHEATH OF CORN DURING GROWTH

Length of leaf sheath in mm.	Peroxidase activity (moles $\times 10^{-6}$ guaiacol oxidized $\times \text{min.}^{-1} \times \text{mg. protein}^{-1}$) Peroxidase fraction					
	A ₁	A ₂	C ₄	C ₃	C ₂	C ₁
12	0.033	0.031	0.40	1.25	0.15	0.041
26	0.055	0.073	0.50	1.00	0.32	0.078
46	0.060	0.17	0.47	1.00	0.56	0.13
65	0.029	0.30	1.16	0.89	0.69	0.20
73	0.041	0.54	1.90	0.91	0.38	0.11

Much more needs to be known of the substrate specificities, the effect of the medium on the activities of the peroxidases, and the conditions within the plant to determine whether the peroxidases of the leaf sheath mediate the same reaction *in vivo* and are multiple forms of the same enzyme.

Changes in the peroxidase complement during growth. Over a period of 5 days the sheath of the second leaf of corn expands from a length of 10 mm. to 70 mm. in the normal segregant of the dwarf-1 stock used. The specific activity of each peroxidase as a function of the length of the leaf sheath is shown in TABLE 2. The activity of each peroxidase is based on the amount of protein applied to the starch block.

A₁, A₂, C₁, and C₂ increased during elongation of the tissue. C₃ was highest in the unexpanded leaf sheath but decreased in activity during elongation. C₄ increased slightly during elongation but increased sharply as elongation ceased.

In cells of the provascular strands of *Vicia faba* (Jensen, 1955) and the trichoblasts of *Phleum* (Avers and Grimm, 1959) enzymic differentiation precedes morphological change, and the cells differ markedly in peroxidase activity from the surrounding tissue. The unexpanded leaf sheath is relatively homogeneous cytologically and shows the simplest peroxidase pattern with C₃ predominant.

With elongation, cell expansion and differentiation occur, and the peroxidase pattern grows in complexity. These changes suggest that enzymic differentiation also may occur here and that the peroxidase complement of the cell may vary from vascular element to parenchyma and with the developmental stage of each. Since the peroxidases show differences in substrate specificity, an histochemical localization of peroxidase activity with a variety of substrates may show differences in the distribution of the peroxidases among the cells of the leaf sheath.

The effects of dwarfism and gibberellic acid. The effects of dwarfism and the application of gibberellic acid (GA) were studied in the sheath of the second

TABLE 3
THE EFFECT OF DWARFISM AND GIBBERELIC ACID ON THE PEROXIDASES
OF THE CORN LEAF SHEATH

	Length of leaf sheath in mm.	Peroxidase activity (moles $\times 10^{-6}$ guaiacol oxidized $\times \text{min.}^{-1} \times \text{mg. protein}^{-1}$) Peroxidase fraction					
		A ₁	A ₂	C ₄	C ₃	C ₂	C ₁
Dwarf-1							
Dwarf	22	0.027	0.15	0.66	0.90	0.61	0.10
Dwarf + GA	62	0.040	0.25	1.6	1.3	0.90	0.17
Normal	68	0.035	0.35	1.2	0.95	0.69	0.20
Normal + GA	77	0.030	0.25	1.1	0.98	0.57	0.12
Dwarf-3							
Dwarf	22	0.027	0.075	0.59	1.2	0.20	0.047
Dwarf + GA	66	0.039	0.14	1.8	1.2	0.27	0.053
Normal	90	0.047	0.22	1.3	1.4	0.20	0.050
Normal + GA	93	0.036	0.17	1.3	1.5	0.21	0.065
Dwarf-5							
Dwarf	12	0.029	0.16	1.0	3.5	0.49	0.061
Dwarf + GA	59	0.045	0.24	1.4	2.2	0.34	0.11
Normal	75	0.074	0.30	1.3	2.5	0.38	0.062
Normal + GA	81	0.040	0.23	1.4	1.9	0.34	0.027
Dwarf-8							
Dwarf	21	0.024	0.18	0.77	0.90	0.42	0.066
Dwarf + GA	19	0.025	0.17	0.74	0.75	0.40	0.056
Normal	80	0.031	0.31	1.3	0.72	0.47	0.059

leaf in four single gene nonallelic dwarf mutants (d_1 , d_3 , d_5 , D_8). The length of the leaf sheath and the specific activity of each peroxidase are shown in table 3 for the dwarf, normal, and GA-treated plants.

In each dwarf growth was reduced, but the dwarfs differed from one another in amount of growth (20 mm. in d_3 , 12 mm. in d_5) and in the degree of growth inhibition (65 per cent in d_1 , 85 per cent in d_5). In all dwarfs the peroxidase complement differed from that of the nondwarf segregant. The activity of A_2 and C_4 was reduced in the dwarfs, but changes in the other peroxidases depended on the particular mutant. C_2 was increased in d_5 but decreased in d_1 . C_3 was increased in both d_5 and D_8 . The normal segregants of the different seed stocks were not equivalent in growth or peroxidase complement. The activity of C_4 was the most constant but the activities of the other peroxidases in the nondwarf depended on the seed stock used.

Treatment with gibberellic acid (K gibberellate 75 per cent*) increased growth in d_1 , d_3 , and d_5 but not in D_8 . In D_8 there was no effect of GA but in the other dwarfs GA reversed the effect of dwarfism on the peroxidase pattern. In d_1 and d_3 GA increased the level of C_4 above that of the nondwarf.

Phinney and West (1960) have suggested that in the recessive d_1 , d_3 , and d_5 mutants a metabolic chain is broken previous to the link represented by gibberellic acid. In D_8 the chain may be broken subsequent to GA or another substance may be involved which is not related to the gibberellins. A comparison of dwarf and normal, GA-treated and untreated, indicates that the level of gibberellic acid in the plant exerts a marked effect upon the peroxidases of the leaf sheath as well as on growth. The differences among the dwarfs in growth and peroxidase complement suggest that not only a lack of GA but also the position of the block on the pathway may be important and have an effect on the plant. The differences of the nondwarf plants in peroxidase pattern and growth among the various seed stocks indicate the presence of genetic factors other than the dwarf ones studied that affect growth and peroxidase patterns and vary from seed stock to seed stock. Thus it is also possible that the differences among the dwarfs are due to a modification of the dwarf trait by these unknown factors.

Every change in growth was accompanied by a change in the peroxidase pattern, but whether an altered peroxidase pattern is a cause or consequence of altered growth cannot be determined yet. A comparison of the changes with development and the effects of dwarfism and GA on the peroxidases shows that it may not be possible to predict the role of an enzyme merely by correlating changes in its activity with the changes in the growth of the tissue. In the elongating leaf sheath the activity of C_4 rose as the growth rate decreased. In all dwarfs the activity of C_4 was decreased, but it increased upon treatment with GA. Thus C_4 is correlated with both decreased and increased growth. Perhaps the contradiction could be resolved by ceasing to equate growth with an increase in length. Growth is the result of cell division, elongation, and differentiation; perhaps a morphological study of the growth coupled with histochemical and enzymic studies of the peroxidases of the leaf sheath may reveal the physiological role of peroxidase and the significance of multiple forms of the same enzyme.

Summary

Six peroxidase active fractions were obtained from corn-leaf sheath preparations by starch electrophoresis. The four major fractions differed in substrate specificity and in the effect of 2,4-dichlorophenol and $MnCl_2$ upon their IAA oxidase activity. The peroxidase pattern of the sheath of the second leaf changed as the tissue elongated and matured. In four dwarf mutants, d_1 , d_3 , d_5 and D_8 , the peroxidase pattern differed from that of the nondwarf segregants. Gibberellic acid promoted growth in the d_1 , d_3 , and d_5 and reversed the effect of dwarfism on the peroxidase pattern.

* Nutritional Biochemicals Corp.

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References

- AVERS, C. J. & R. B. GRIMM. 1959. Comparative enzyme differentiation in grass roots. II. Peroxidase. *J. Exptl. Botany*. **10**: 341-344.
- AZAKAWA, T. & E. E. CONN. 1958. The oxidation of reduced pyridine nucleotides by peroxidase. *J. Biol. Chem.* **232**: 403-415.
- BURRIS, R. H. 1960. Hydroperoxidases (peroxidases and catalases). : 365-400. *In* W. Ruhland, Ed. *Encyclopedia of Plant Physiology*, XII (1). Springer-Verlag. Berlin, Germany.
- CHANCE, B. 1952. Oxidase and peroxidase reactions in the presence of dihydroxymaleic acid. *J. Biol. Chem.* **197**: 577-589.
- CHANCE, B. 1954. Enzyme mechanisms in living cells. : 399-460. *In* W. D. McElroy & B. Glass, Eds. *A Symposium on the Mechanism of Enzyme Action*. Johns Hopkins Press. Baltimore, Md.
- GALSTON, A. W., J. BONNER & R. S. BAKER. 1953. Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. *Arch. Biochem. Biophys.* **42**: 456-470.
- GALSTON, A. W. & L. Y. DALBERG. 1954. The adaptive formation and physiological significance of indoleacetic acid oxidase. *Am. J. Botany*. **41**: 373-380.
- GALSTON, A. W. & D. C. MCCUNE. 1961. An analysis of gibberellin-auxin interaction and its possible metabolic basis. : 611-625. *In* R. M. Klein, Ed. *4th International Conference on Plant Growth Regulation*, Yonkers, N.Y., 1959. Iowa State Univ. Press. Ames, Iowa.
- GARAY, A. S. & M. FARAGÓ. 1959. Papierelektrophoretische Trennung Peroxydase-wirkender Agenten aus Pflanzen. *Phyton*. **13**: 55-57.
- HIGUCHI, T. 1957. Biochemical studies of lignin formation. III. *Physiol. Plant.* **10**: 633-648.
- HOSOYA, T. 1960a. Turnip peroxidase. I. Purification and physicochemical properties of multiple components in turnip peroxidase. *J. Biochem.* **47**: 369-381.
- HOSOYA, T. 1960b. Turnip peroxidase. II. The reaction mechanisms of turnip peroxidases A₁, A₂ and D. *J. Biochem.* **47**: 794-803.
- HOSOYA, T. 1960c. Turnip peroxidase. IV. The effect of pH and temperature upon the rate of reaction. *J. Biochem.* **48**: 178-189.
- JENSEN, W. A. 1955. The histochemical localization of peroxidase in roots and its induction by indoleacetic acid. *Plant Physiol.* **30**: 426-432.
- JERMYN, M. A. & R. THOMAS. 1954. Multiple components in horse-radish peroxidase. *Biochem. J.* **56**: 631-639.
- KAMERBEEK, G. A. 1956. Peroxydase content of dwarf types and giant types of plants. *Acta Bot. Neerl.* **5**: 257-263.
- KENTEN, R. H. & P. J. G. MANN. 1953. The oxidation of certain dicarboxylic acids by peroxidase systems in presence of manganese. *Biochem. J.* **53**: 498-505.
- KONDO, K. & Y. MORITA. 1952. Phytoperoxidase. II. Isolation and purification of sweet-potato peroxidases and their absorption spectra. *Bull. Research Inst. Food Sci. Kyoto Univ.* **10**: 33-45.
- KONISHI, M. 1956. Studies on development of flowering stalks in long-day plants in relation to auxin metabolism. *Mem. College Agric. Univ. Kyoto*. **75**: 1-70.
- LAYNE, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. : 447-454. *In* S. P. Colowick & N. O. Kaplan, Eds. *Methods in Enzymology*. III. Academic Press. New York, N.Y.
- MCCUNE, D. C. & A. W. GALSTON. 1959. Inverse effects of gibberellin on peroxidase activity and growth in dwarf strains of peas and corn. *Plant Physiol.* **34**: 416-418.
- MARKERT, C. L. & F. MØLLER. 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci.* **45**: 753-763.
- MUDD, J. B. & R. H. BURRIS. 1959. Participation of metals in peroxidase-catalyzed oxidations. *J. Biol. Chem.* **234**: 2774-2777.
- PAUL, K. G. 1958. Die Isolierung von Meerrettichperoxydase. *Acta Chem. Scand.* **12**: 1312-1318.

- PHINNEY, B. O. & C. A. WEST. 1960. Gibberellins as native plant growth regulators. *Ann. Rev. Plant Physiol.* **11**: 411-436.
- RAY, P. M. 1956. The destruction of indoleacetic acid. II. Spectrophotometric study of the enzymic reaction. *Arch. Biochem. Biophys.* **64**: 193-216.
- RAY, P. M. 1960. The destruction of indoleacetic acid. III. Relationships between peroxidase action and indoleacetic acid oxidation. *Arch. Biochem. Biophys.* **87**: 19-30.
- SIEGEL, S. M. 1956. Chemistry and physiology of lignin formation. *Quart. Rev. Biol.* **31**: 1-18.
- STUMPF, P. K. & C. BRADBEER. 1959. Fat metabolism in higher plants. *Ann. Rev. Plant Physiol.* **10**: 197-222.

THE CHROMATOGRAPHIC FORMS OF CYTOCHROME *c**

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Proteins showing the characteristic hemochromogen spectrum that classifies them as cytochromes *c* have been prepared from a large variety of animals, plants, and microorganisms, and exhibit a correspondingly wide range of properties. For example their isoelectric points vary from the acidic to the strongly basic range, their molecular weights from nearly 100,000 to 12,000 and their oxidation-reduction potentials from +0.320 V to -0.204 V.¹⁻³

In the present short review of the evidence relevant to the possible physiological occurrence of more than a single molecular form of any one particular cytochrome *c* the discussion will be limited to the mammalian type of cytochromes *c*. These can be defined as those cytochromes *c* which are readily enzymically oxidized by cytochrome oxidase preparations obtained from mammalian sources. Such cytochromes *c* have been isolated from mammals (horse, cow, pig, whale, and others),⁴⁻⁸ birds (chicken, King penguin, pigeon),⁹⁻¹¹ fish (tunny, bonito),¹² wheat germ,^{13,14} yeast,^{15,16} and a bacterium, *Micrococcus denitrificans*.¹⁷ Many of them have been crystallized during the last five years.^{6-8,10-12,14,16} Every one of these proteins that has been examined was found to have a molecular weight of about 12,000,¹⁸ an unusually basic isoelectric point¹⁹ and an oxidation-reduction potential of about +0.250 V.²⁰

Results that may be interpreted as evidence for the inhomogeneity of cytochrome *c* preparations have come from ion-exchange chromatography, low temperature spectra, electrophoresis, and the separation of more than one hemin from the protein. These observations will be examined in turn, attempting to correlate them with the possible state of cytochrome *c* *in vivo*.

Ion Exchange Chromatography

Cytochromes *c* of the mammalian type with their relatively low molecular weight and high basicity are particularly well suited to column chromatography on weak cation exchangers. A chromatographic procedure for the purification of cytochrome *c* was first introduced by Paléus and Neilands²¹ and subsequently modified to yield the undenatured protein.¹⁸ Amberlite IRC-50,† a polycarboxylic resin, has been used chiefly for the chromatography of cytochrome *c* although other weak cation exchangers such as Decalso F²²‡ and celite ion-exchangers²³ are just as effective. Boardman and Partridge²⁴ studied quantitatively the chromatographic behavior of horse-heart cytochrome *c* on columns of Amberlite IRC-50, showing that the protein is adsorbed irreversibly by short-range forces below *pH* 6; in the neutral *pH* range the effective forces are largely electrostatic depending both on the ionization of the resin, which is depressed as the *pH* decreases, and the basic ionization of the protein, which

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† Rohm and Haas Co., Philadelphia, Pa.

‡ Permutit Co. Ltd., London, England.

is depressed as the pH increases. In the intermediate range a true ion-exchange process results, while at pH values of 10 and above where the ϵ -amino groups of lysyl residues on the protein lose their positive charge the protein is not absorbed by the resin. Thus in the pH range in which an ion-exchange process is dominant the mobility of any particular form of cytochrome c under specified conditions of temperature and ionic strength will depend largely on the number of basic groups on the protein available for reaction with the cation-exchanger.

The classical procedure for the preparation of cytochrome c ²⁵ involves an initial extraction with acid, trichloroacetic acid being the one most commonly used. When such preparations are chromatographed on cation exchangers more than one fraction is invariably observed. Paléus and Neilands²¹ obtained three fractions. Margoliash¹⁸ and Margoliash *et al.*,²⁶ showed that one could separate numerous fractions depending on the number of steps by which the cation concentration was increased during the chromatography. Only one of these fractions, that eluted at a cation concentration of 0.22 to 0.25 gm. ion/l.^{18, 24, 26} can be considered to approximate the native protein since it has maximal enzymic activity and the typical, highly specific hemochromogen properties of cytochrome c . Indeed this material termed fraction I²⁶ is not autoxidizable, does not combine significantly with carbon monoxide at neutral pH and has a spectrum in the reduced form that is unaffected by the hydrogen ion concentration from pH 3 to pH 12. All the other fractions collectively termed fractions II are eluted only at higher ionic strengths, have hemochromogen properties that differ from those of the native material or fraction I, and clearly represent denatured or partially unfolded forms of the protein.²⁶ The order in which these fractions move off the column is in fact the order in which all of the hemochromogen properties of these preparations vary, from those nearest to native cytochrome c for those fractions eluted at cation concentrations nearest those that elute the native protein to those essentially indistinguishable from ordinary chemical hemochromogens for those fractions eluted at the highest cation concentrations used.^{26, 27}

It was moreover shown that the original acid extraction step was responsible for the presence of the fractions II. Prolonging the acid extraction increases the yield of fraction II, while the use of neutral salt extractions diminishes its amount to the vanishing point,¹⁸ without decreasing the total yield of cytochrome c .²⁷ Fraction I can readily be transformed into a mixture of fractions II by treatment with acids¹⁸ or organic solvents such as ethanol and acetone.²⁷ The yield of the various fractions II in such experiments depends on the extent to which the denaturation procedures are applied.²⁷ Prolonged denaturation in ethanol gives preparations that contain chiefly material eluted at concentrations of 1.0 gm. ion/l., while milder treatments result mainly in materials which can be eluted at about 0.5 gm. ion/l. FIGURE 1 shows a chromatogram of a mildly denatured total fraction II, the elution being performed under a linear sodium ion concentration gradient. Each individual subfraction II can be denoted by the cation concentration at which its chromatographic peak appears under such conditions. When subfraction II_{0.5}, for example, is rechromatographed using a 0.5 gm. ion/l. solvent it runs as a homogeneous substance of fixed chromato-

graphic properties and can readily be separated from the contaminant adjoining fractions as seen in FIGURE 2.

It should however be noted that more recent evidence indicates that even those preparations eluted at 0.25 gm. ion/l. may contain a small proportion of partially denatured material. Morrison *et al.*²⁸ have found that such fractions I

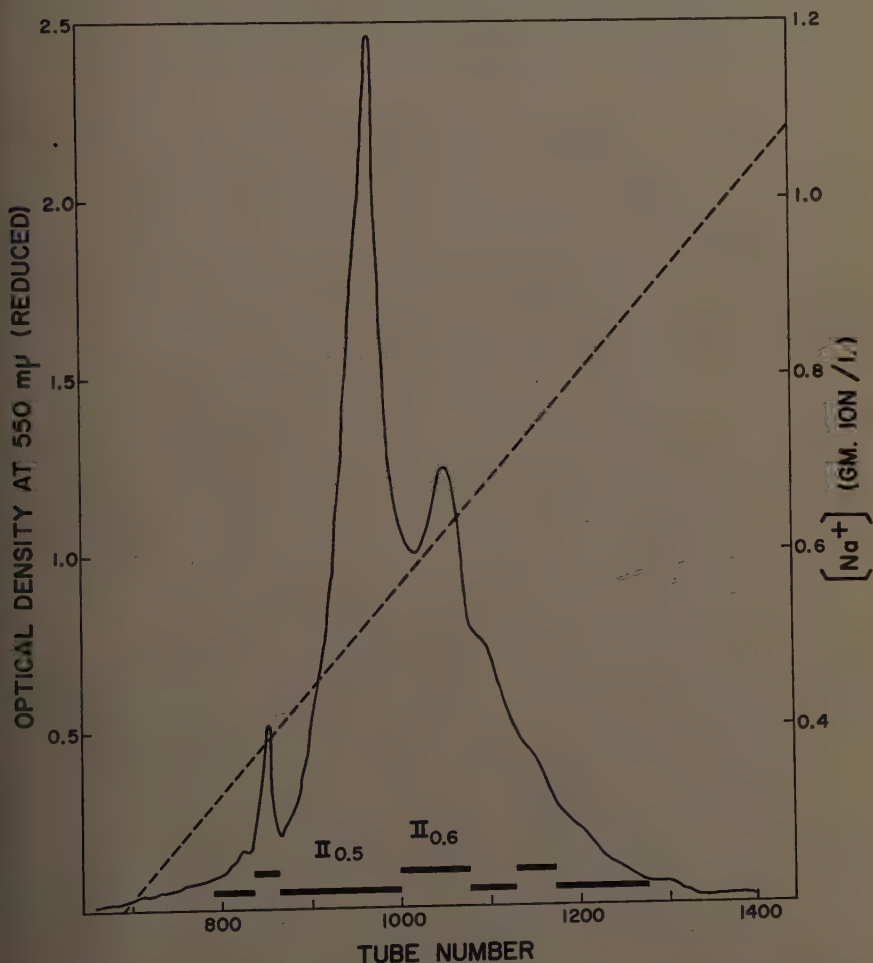


FIGURE 1. Chromatography on Amberlite IRC-50 of a mildly ethanol denatured horse heart cytochrome *c*. Chromatographic fraction I was kept in 66 per cent ethanol for 1 hour at room temperature; the total fraction II formed was separated by chromatography and rechromatographed at pH 7.0 (0.02 *M* phosphate buffer) under a linear $[Na^+]$ gradient (NaCl). The total $[Na^+]$ was determined in the effluent fractions by flame photometry (dashed line; ordinate on the right). The solid line represents the concentration of cytochrome *c* in the fractions (optical density at 550 mμ, reduced form; ordinate on the left). The heavy lines above the abscissa indicate the fractions pooled. Amberlite IRC-50 (200–400 mesh) column in Na^+ form: 3.8×150 cm. 3.0 gm. of fraction II was applied. Flow rate: 150 ml./hour; temperature, 4° C.; 15.5 ml. fractions were collected.

separated from acid extracted preparations of the protein have slightly lower enzymic activities than similar fractions made from salt extracted preparations.

An entirely different type of modified cytochrome *c* is obtained when denaturation is carried out in alkali. This process is accompanied by the loss of amide groups from the protein and results in a material that is not adsorbed onto Amberlite IRC-50 under the usual conditions.²⁷ Occasionally such products are formed on the cation exchanger columns when the resin has not been properly equilibrated with the eluting buffer or when chromatography is carried out at pH 9 and above.²⁷

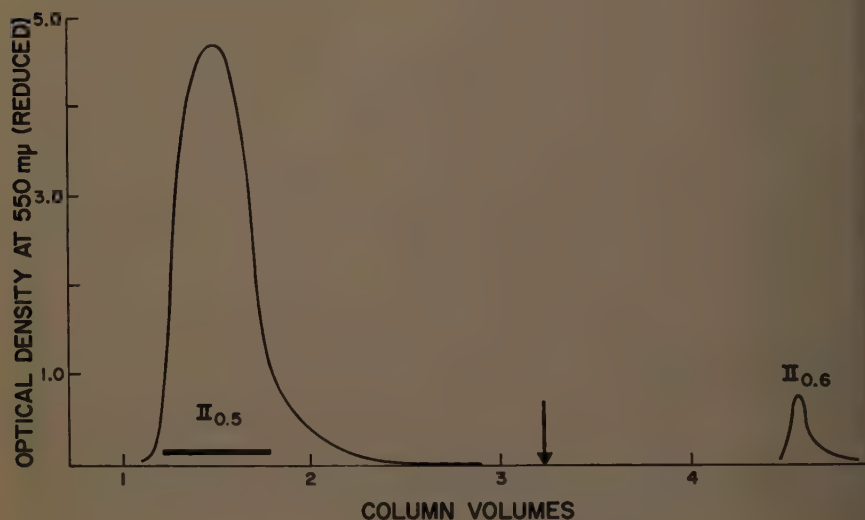


FIGURE 2. Rechromatography of subfraction $II_{0.5}$ on Amberlite IRC-50. The conditions were the same as in FIGURE 1 except that the flow rate was 60 ml./hour and the eluting solution contained a $[Na^+]$ of 0.495 gm. ion/l. At the arrow this solution was changed to one of 0.600 gm. ion/l. Na^+ . About 65 per cent of fraction $II_{0.5}$ collected in the chromatography shown in FIGURE 1 was applied.

To complicate the chromatographic picture further cytochrome *c* in the reduced form, which has one positive charge less than the oxidized form, will readily separate from the latter.^{18,21} To avoid such complications, particularly since impurities in the resins often result in a certain degree of reduction of the protein on the column,¹⁸ it is advisable to add a small amount of an oxidizing agent such as potassium ferricyanide to the eluting solutions.⁷

In conclusion, the chromatographic evidence indicates that preparations of cytochrome *c* contain a single native form of the protein that is presumably the one occurring *in vivo*. The other chromatographic forms observed represent variously denatured materials that are artifacts of the preparation. An essentially similar conclusion was reached by Yamanaka *et al.*²⁹ on the basis of a study of some properties of native and modified cytochrome *c* from various mammalian sources.

Low Temperature Spectra

Keilin and Hartree³⁰ first observed that when cytochrome *c* is cooled to the temperature of liquid nitrogen in a water-glycerol medium, the characteristic α and β absorption bands of the reduced hemochromogen are sharpened, intensified and split into several components. More recently Estabrook^{31,32} has recorded spectrophotometrically the low temperature spectra of a variety of cytochromes *c* showing that the α band is split into three satellite bands³⁰ and the β band into nine. This phenomenon could be taken to indicate the existence of more than one type of hemochromogen in the preparations and thus, presumably, more than one molecular form of the protein.³³

However, an examination of increasingly acid-denatured chromatographic fractions of horse heart cytochrome *c* reveals that the characteristic splitting of the absorption bands becomes less and less distinct as the degree of disorganization of the native structure is more pronounced.³² A cytochrome *c* "hemopeptide" obtained by digestion with pepsin and containing the heme with only 11 adjacent amino acids^{34,35} as well as alkaline denatured cytochrome *c* both show a single symmetrical α band. It is therefore probable that the multiple α bands of the low-temperature spectra do not represent several molecular species but are rather due to the resolution of vibrational components of the electronic transition resulting in the α band and that, moreover, the absorption bands are profoundly influenced by the conformation of the protein.³² That the conformation of the protein has a determining influence on the reactivity of the cytochrome *c* hemochromogen was already indicated by the studies cited above on the properties of denatured chromatographic fractions.²⁶

Of the five cytochromes *c*, the low temperature spectra of which were investigated by Estabrook,³² yeast cytochrome *c* is the only one that does not show the fine structure of the α band. In a number of other respects crystalline yeast cytochrome *c* has been shown to have the characteristics of denatured forms of other cytochromes *c*; it travels on cation exchanger columns at the high solvent cation concentrations required to elute denatured mammalian cytochromes *c*³⁶ although its amino acid composition is very similar to that of the beef or horse heart proteins,^{37,38} moreover yeast cytochrome *c* is more readily digested by proteolytic enzymes than even denatured beef heart cytochrome *c*³⁹ whereas the native forms of other cytochromes *c* are distinctly more resistant to proteolytic digestion than the corresponding denatured proteins.²⁹ The various chromatographic fractions reported in preparations of yeast cytochrome *c*^{37,39} most probably correspond to various degrees of denaturation of the protein, but whether the crystalline preparations already represent a protein differing in conformation from the native material as it is present in the cell cannot be decided. On the other hand all these peculiarities could be due to a different hemochromogen structure in yeast cytochrome *c* as compared to the other cytochromes *c* of the mammalian type. Indeed, as discussed below, not only does the conformation of the protein affect the properties of the hemochromogen but, conversely, the state of oxidation or reduction of the hemochromogen has distinct effects on properties that can be ascribed only

to the protein. In this respect it is interesting to note that the peptide chains of yeast and silkworm cytochrome *c* both contain an arginyl residue adjacent to one of the two cysteinyl residues that are bonded to the heme through thioether links, replacing a lysine found in the same position in other cytochromes *c*.⁴⁰ As far as we are aware the low temperature spectrum of silkworm cytochrome *c* has not been examined so that the probability of the suggested correlation cannot be judged at present.

Electrophoresis

Clear-cut evidence for the inhomogeneity of cytochrome *c* preparations has come from a study by Paléus and Theorell⁴¹ of the electrophoretic behavior of crystalline beef heart cytochrome *c*. The protein crystallized by two somewhat different procedures separated into three fractions. The main fraction crystallized more easily than the original material. The enzymic and chemical hemochromogen properties, the low temperature spectra and the possible relationships of these fractions to the usual chromatographic fractions already discussed have not been studied. It is therefore not possible to decide whether the multiple electrophoretic species represent true *in vivo* variants of the protein or are simply conformational variants resulting from the preparation procedures.

The Isolation of More Than One Hemin from Cytochrome c

When hematohemin is prepared from a chromatographically purified cytochrome *c* by the silver salt procedure of Paul,⁴² which splits the two thioether bonds binding the heme to the peptide chain,⁴³ the resulting material contains at least two components^{28, 44-46} that can be separated either by column or paper chromatography. The silver salt procedure involves rather drastic conditions of acidity and temperature and could conceivably result in oxidation or, even more likely, in isomerization reactions leading to either α or β attachment of the hydroxyl groups in one or both of the appropriate hematohemin side chains. In cytochrome *c* the sulfur atoms of the two thioether bridges are almost certainly attached to the α carbon of the vinyl protoporphyrin side chains.^{33, 47-49} Since the exact structure of the hematohemin fractions is unknown and the splitting of thioether links by silver salts has not been studied on the appropriate model synthetic cytochrome *c* heme⁴⁹ as regards the possible yield of more than one hematohemin fraction, it cannot be decided whether these experiments do in fact show the existence of more than one molecular form in the original preparation of the protein. It should be noted that two hematohemin fractions can be obtained whether a native or denatured cytochrome *c* is used.⁴⁵

Chromatographic Fractions and the Relation of Hemochromogen Properties to Protein Conformation

The demonstration that the various chromatographic species of cytochrome *c* separated on cation exchanger columns were conformational variants of the native molecule has provided a useful tool for studying the relationships existing between the structure of the protein and the properties of the active site.

If one assumes that the separation of the various species under the appropriate chromatographic conditions discussed above is due solely to ion exchange phenomena it follows that those fractions that are eluted at cation concentrations higher than those required for the native protein contain a larger number of reactive basic groups than the native protein, indicating various degrees of disorganization or "unfolding" of the molecule. It should be noted that all the fractions II contain the full complement of amino acids present in native cytochrome *c*.

The properties of the active site of cytochrome *c* are not those of an ordinary chemical hemochromogen but are abnormal. Thus the spectrum of the ferro form is not *pH*-dependent over a wide range extending to far below the *pK'* of the possible basic groups involved in hemochromogen formation; the heme in the native protein is not autooxidizable and does not react with carbon monoxide, and cytochrome *c* reacts with appropriate enzyme systems of the terminal oxidation chain. These and other specific properties that distinguish cytochrome *c* from simple chemical hemochromogens have been interpreted in terms of a "crevice" structure of the protein in which the heme is inbedded and tightly held.⁵⁰ George and Lyster⁵⁰ have defined the requirements of such a crevice structure and the physicochemical parameters affected by its presence.

Since the degree of disorganization of the native structure reflected in the number of basic groups available for reaction with cation exchangers is in direct proportion to the extent in which the properties of the active site differ from those of the native protein and approximate those of a simple hemochromogen, it appears reasonable to conclude that it is the entire native configuration of the protein that imparts to the active site its peculiar properties by maintaining the structural integrity of the crevice. Disturbances of the native conformation appear to have quantitative rather than qualitative effects on the rigidity of this structure. Partially unfolded molecules have partial enzymic activities and the whole spectrum of properties described above.

There is however one possible fallacy in this argument that should be made clear. The denatured fractions II separated thus far do not necessarily represent preparations of unimolecular species but are most probably statistical mixtures of molecules having the same range of reactive cationic sites. Thus if one were to assume, in opposition to the conclusion presented above, that a single small area in the protein is entirely responsible for the properties of the crevice, it is possible that each of the denatured chromatographic fractions contains a proportion of molecules disorganized in this particular area in exact proportion to the amount of disorganization in other areas of the protein. Such a situation is unlikely in view of the fixed chromatographic characteristics of the various fractions II under a fairly wide range of conditions and the grossly nonuniform distribution of basic residues in the amino acid sequence of the protein.⁵¹ Whatever the nature of the bonds holding together the native configuration of the protein the breakage of each one must entail the opening or disorganization of a particular area of the protein. It is also not probable that many such structural interfold stabilizing bonds in the center of the structure could be broken without having bonds on the outside of the structure or the ends of the peptide chain also opened. Thus since the basic residues are unequally distributed along the chain,⁵¹ the results discussed above on the

relation of the degree of denaturation to the properties of the hemochromogen in cytochrome *c* would be unlikely if a small area of the protein were alone responsible for the integrity of the crevice and the abnormal hemochromogen properties.

Not only is the conformation of the protein important in maintaining the peculiar properties of the hemochromogen but, conversely, the state of oxidation or reduction of the hemochromogen affects the properties of the protein. This remarkable phenomenon has long been known from the studies of Jonxis⁵² who in 1939 demonstrated that ferri-cytochrome *c* spreads more rapidly into a monomolecular layer than reduced cytochrome *c* at a water-air interface. More recently Nozaki *et al.*^{29,39,53,54} have found that reduced cytochrome *c* is more slowly digested by proteolytic enzymes than the oxidized form. Ferro-cytochrome *c* also displays different crystal forms from those of ferricytochrome *c*^{7,11,12,14} and is probably more compact than the latter.

The conclusion as to the requirement of at least a large proportion of the native configuration of the protein for imparting to the cytochrome *c* hemochromogen its specific properties appears well founded as far as the available evidence indicates. That the total complement of amino acids is nevertheless not required was shown by Titani *et al.*,⁵⁵ since a digestion with carboxypeptidase that probably removed the last four amino acids C-terminal to a lysyl-lysyl sequence⁵¹ did not have any measurable effect on the enzymic properties of the protein. The exact influence of various portions of the native structure on the properties of the active site, however, will be established only when preparations of unimolecular species of denatured cytochrome *c* become available and are chemically defined as to which particular disorganized areas they contain. A complication in the interpretation of the properties of the denatured fractions comes from the demonstration of Nozaki⁵⁶ that thoroughly acid denatured preparations of cytochrome *c* exist in solution as dimers. The importance of this observation with regard to the above conclusion cannot as yet be assessed.

The properties of cytochrome *c* discussed above refer only to its electron transfer or oxidoreduction functions. Preparations having intermediate activities with respect to this group of properties can readily be obtained. This does not appear to be the case with regard to the involvement of the protein in the oxidative phosphorylation reactions of mitochondrial preparations. Thus Morrison *et al.*⁵⁷ find that an acid denatured cytochrome *c*, although it partly retained its ability to stimulate the oxidation of ascorbate by rat liver mitochondria, was completely incapable of sustaining oxidative phosphorylation.

Conclusions and Summary

The observations relevant to the possible existence of more than one molecular form of the mammalian type of cytochrome *c* reviewed in this paper, have indeed shown the presence of relatively numerous molecular species in preparations of these proteins, whether amorphous or crystalline. In the case of the various fractions separable by cation exchanger chromatography a study of their properties has shown that they represent only conformational variants of an apparently single native structure arising as a result of preparative pro-

cedures. These fractions thus give no evidence of the existence of more than one molecular form of cytochrome *c* *in vivo* in any single tissue. A similar conclusion can be drawn from the study of the low temperature spectra. In the other types of experiments discussed the properties or structure and mode of formation of the products have not been examined, so that it cannot be judged whether these observations do or do not indicate the existence of more than one natural molecular form of the protein.

We feel that the case of cytochrome *c*, in so far as it has been worked out, provides a valuable example of one of the pitfalls attending the demonstration of the actual physiological occurrence of multiple molecular forms of one protein, whether it has or does not have a known enzymic function. No single procedure of separation can give such proof, even if the experimental conditions are adequately controlled from the point of view of avoiding known artifacts. It is essential in every case to provide a clear-cut demonstration that the various postulated molecular forms either cannot conceivably or do not in fact arise one from the other as a result of the experimental procedures. Two types of such demonstrations appear to be unequivocal: in one, a definite structural difference, such as for example in the sequence of the amino acids, is shown to occur; in the other, the different molecular forms are proved to be products of separate biosynthetic pathways under independent genetic control.

References

1. NEWTON, J. W. & M. D. KAMEN. *In The Bacteria*. R. Y. Stanier & I. C. Gunsalus, Eds. Academic Press. New York, N.Y. In press.
2. KAMEN, M. D. *In Symposia in Comparative Biology*. M. B. Allen, Ed. Academic Press. New York, N.Y. In press.
3. KAMEN, M. D. *In Proceedings of the Symposium on Biological Structure and Function*. Stockholm, Sweden. In press.
4. KEILIN, D. & E. F. HARTREE. 1937. *Proc. Roy. Soc. (London)*. **122B**: 298.
5. THEORELL, H. 1936. *Biochem. Z.* **285**: 207.
6. HAGIHARA, B., I. MORIKAWA, I. SEKUZU, T. HORIO & K. OKUNUKI. 1956. *Nature*. **178**: 630.
7. HAGIHARA, B., I. MORIKAWA, I. SEKUZU & K. OKUNUKI. 1958. *J. Biochem. (Tokyo)*. **45**: 551.
8. MINAKAMI, S., K. TITANI, H. ISHIKURA & K. TAKAHASHI. 1958. *J. Biochem. (Tokyo)*. **45**: 547.
9. PALÉUS, S. 1954. *Acta Chem. Scand.* **8**: 971.
10. BODO, G. 1955. *Nature*. **176**: 829.
11. HAGIHARA, B., M. YONEDA, K. TAGAWA, I. MORIKAWA & K. OKUNUKI. 1958. *J. Biochem. (Tokyo)*. **45**: 565.
12. HAGIHARA, B., K. TAGAWA, I. MORIKAWA, M. SHIN & K. OKUNUKI. 1958. *J. Biochem. (Tokyo)*. **45**: 725.
13. GODDARD, D. R. 1944. *Am. J. Botany*. **31**: 270.
14. HAGIHARA, B., K. TAGAWA, I. MORIKAWA, M. SHIN & K. OKUNUKI. 1959. *J. Biochem. (Tokyo)*. **46**: 321.
15. KEILIN, D. 1930. *Proc. Roy. Soc. (London)*. **106B**: 418.
16. HAGIHARA, B., T. HORIO, J. YAMASHITA, M. NOZAKI & K. OKUNUKI. 1956. *Nature*. **178**: 629.
17. KAMEN, M. D. & L. P. VERNON. 1955. *Biochim. et Biophys. Acta*. **17**: 10.
18. MARGOLIASH, E. 1954. *Biochem. J.* **56**: 535.
19. THEORELL, H. & Å. ÅKESSON. 1941. *J. Am. Chem. Soc.* **63**: 1804.
20. HENDERSON, R. W. & W. A. RAWLINSON. 1956. *Biochem. J.* **62**: 21.
21. PALÉUS, S. & J. B. NEILANDS. 1950. *Acta Chem. Scand.* **4**: 1024.
22. MARGOLIASH, E. 1954. *Biochem. J.* **56**: 529.
23. BOARDMAN, N. K. 1959. *J. Chromat.* **2**: 388.
24. BOARDMAN, N. K. & S. M. PARTRIDGE. 1953. *Nature*. **171**: 208.
25. KEILIN, D. & E. F. HARTREE. 1945. *Biochem. J.* **39**: 289.

26. MARGOLIASH, E., N. FROHWIRT & E. WIENER. 1959. *Biochem. J.* **71**: 559.
27. MARGOLIASH, E. & J. LUSTGARTEN. Unpublished data.
28. MORRISON, M., T. HOLLOCHER, R. MURRAY, G. MARINETTI & E. STOTZ. 1960. *Biochim. et Biophys. Acta.* **41**: 334.
29. YAMANAKA, T., H. MIZUSHIMA, M. NOZAKI, T. HORIO & K. OKUNUKI. 1959. *J. Biochem. (Tokyo)*. **46**: 121.
30. KEELIN, D. & E. F. HARTREE. 1949. *Nature*. **164**: 254.
31. ESTABROOK, R. W. 1956. *J. Biol. Chem.* **223**: 781.
32. ESTABROOK, R. W. *In Proceedings of the Haematin Enzyme Symposium.* J. E. Falk, M. R. Lemberg & R. K. Morton, Eds. Pergamon Press. London, England. In press.
33. PAUL, K. G. 1960. Heme compounds in enzyme catalysis. *In The Enzymes*. 2nd ed. **3**: 319. P. D. Boyer, H. Lardy & K. Myrback, Eds. Academic Press. New York, N.Y.
34. TSOU, C. L. 1951. *Biochem. J.* **49**: 367.
35. TUPPY, H. & S. PALÉUS. 1955. *Acta Chem. Scand.* **9**: 353.
36. MINAKAMI, S., H. ISHIKURA & K. SATAKE. 1956. *J. Biochem. (Tokyo)*. **43**: 575.
37. NUNNIKHOFEN, R. 1958. *Biochim. et Biophys. Acta.* **28**: 108.
38. TAKAHASHI, K., K. TITANI & S. MINAKAMI. 1959. *J. Biochem. (Tokyo)*. **46**: 1323.
39. NOZAKI, M., T. YAMANAKA, T. HORIO & K. OKUNUKI. 1957. *J. Biochem. (Tokyo)*. **44**: 453.
40. PALÉUS, S. & H. TUPPY. 1959. *Acta Chem. Scand.* **13**: 641.
41. PALÉUS, S. & H. THEORELL. 1957. *Acta Chem. Scand.* **11**: 905.
42. PAUL, K-G. 1950. *Acta Chem. Scand.* **4**: 239.
43. THEORELL, H. 1939. *Enzymologia*. **6**: 88.
44. MORRISON, M., R. W. ESTABROOK & E. STOTZ. 1954. *J. Am. Chem. Soc.* **76**: 6409.
45. STOTZ, E., M. MORRISON & G. MARINETTI. 1956. *In Enzymes: Units of Biological Structure and Function.* : 465. O. H. Gaebler, Ed. Academic Press. New York, N.Y.
46. MORRISON, M. & E. STOTZ. 1957. *J. Biol. Chem.* **228**: 123.
47. PAUL, K-G. 1951. *Acta Chem. Scand.* **5**: 389.
48. GRANICK, S., L. BOGORAD & H. JAFFE. 1953. *J. Biol. Chem.* **202**: 801.
49. NEILANDS, J. B. & H. TUPPY. 1960. *Biochim. et Biophys. Acta.* **38**: 351.
50. GEORGE, P. & R. L. J. Lyster. 1958. *Proc. Natl. Acad. Sci. U. S.* **44**: 1013.
51. MARGOLIASH, E., E. L. SMITH, H. TUPPY & G. KREIL. Unpublished data.
52. JONXIS, J. H. P. 1939. *Biochem. J.* **33**: 1743.
53. NOZAKI, M., H. MIZUSHIMA, T. HORIO & K. OKUNUKI. 1958. *J. Biochem. (Tokyo)*. **45**: 815.
54. MIZUSHIMA, H., M. NOZAKI, T. HORIO & K. OKUNUKI. 1958. *J. Biochem. (Tokyo)*. **45**: 845.
55. TITANI, K., H. ISHIKURA & S. MINAKAMI. 1959. *J. Biochem. (Tokyo)*. **46**: 151.
56. NOZAKI, M. 1960. *J. Biochem. (Tokyo)*. **47**: 592.
57. MORRISON, M., T. HOLLOCHER & E. STOTZ. Unpublished data.

NUCLEAR AND CYTOPLASMIC FACTORS DETERMINING THE SPECIES SPECIFICITY OF ENZYME PROTEINS IN *ACETABULARIA**

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One of the most intriguing problems of modern biology is the nature of the nuclear control of protein synthesis and protein specificity in the cell. Most proteins are synthesized in the cytoplasm although the nucleus also seems to be capable of synthesizing some proteins. Recent biochemical work has characterized in cytoplasmic fractions a number of systems involved in the synthesis of proteins from amino acids. The ribosomes, small ribonucleoprotein particles, are thought to constitute the actual site for the assembly of protein molecules and to carry the "blue print" for protein structure. From genetic experiments evidence has emerged that the structure of many proteins is ultimately determined by nuclear genes. In order to reconcile both these findings one has to postulate the existence of a system that is responsible for the transmittance of genetic information from nuclear genes to the synthetic sites in the cytoplasm.

The rather complex problem of nucleocytoplasmic interactions has been approached experimentally in different ways. One of the most direct approaches is the study of the synthetic capacities of a cell that has been deprived of its nucleus by means of microsurgery. Several metabolic processes can thus be examined with respect to the degree of their dependence upon the presence of a nucleus. Among the few organisms studied in this respect, the unicellular green alga *Acetabularia* occupies a prominent position. The large size of the cells makes handling and microsurgical operations relatively simple. Photosynthesis and respiration continue at normal rates for a long time after enucleation¹ providing carbohydrates and energy-rich phosphates^{2,3} for anabolic processes.

It is now a well-established fact that enucleated plants of *Acetabularia* as well as certain stalk fragments are capable of synthesizing large amounts of protein^{4,5} involving chloroplast protein and other unspecified cytoplasmic proteins.⁶ Moreover, the activities of a number of enzymes investigated, namely aldolase,⁷ invertase, acid phosphatase,⁸ and phosphorylase⁹ were reported to increase anywhere between 40 per cent and 600 per cent after removal of the nucleus. Various enzymes differ in their response in that synthesis becomes impaired at different moments after enucleation, for example, after one week in the case of acid phosphatase. Prior to the period of inhibition the enzyme activities increase at almost parallel rates in enucleated algae and in corresponding nucleate controls, if one takes into account the loss of some cytoplasm during the enucleation operation. It seems that certain unknown substances are used up during the synthesis of various proteins, substances that are replenished by the nucleus under normal conditions. The data ob-

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tained with *Acetabularia* are thus in perfect agreement with Mazia's Replacement Hypothesis.¹⁰

In the present paper still another approach is described concerning the problem of nucleocytoplasmic interactions. As nuclear and cytoplasmic factors might be involved in expressing protein specificity, an attempt has been made to label them differentially by transplanting to an enucleated cytoplasm a species-foreign nucleus. In the resulting graft *de novo* activities of the cell nucleus might be recognizable independently of any genetic information stored in the cytoplasm, provided both species differed significantly in the structure of certain proteins.

Species- and even tissue-specific multiple forms of enzymes (isozymes) have been detected in animal material after separation by starch-gel electrophoresis.^{11,12,13} Because of the sensitivity and the great resolving power of these techniques it seemed of great advantage to make use of the specificity of certain enzyme proteins, as analyzed by electrophoresis, and combine these techniques with nuclear transplantation experiments. Preliminary work with *Acetabularia* had revealed the existence of several species-specific forms of acid phosphatase that differed significantly in their electrophoretic mobilities in starch gel. Maximum differences were encountered between the species *Acetabularia mediterranea* and *Acicularia Schenckii*;¹⁴ since, furthermore, successful grafts between these two species have been reported in the literature,¹⁵ all investigations described in the present paper are restricted to this plant material.

Methods

Starch-gel electrophoresis. Five to 10 algae were ground in small glass homogenizers without the addition of any liquid. The material was transferred to a small piece of filter paper that was then inserted in the starch strip. When individual stalks were to be tested, one plant was placed on a small Lucite block and any residual culture medium removed by careful blotting with filter paper. The rhizoid was cut off and the contents of the stalk squeezed out. A small drop was produced consisting of cytoplasm and cell sap. The material was adsorbed onto a 2 × 5-mm. large piece of filter paper and then inserted in the starch. The techniques described by Smithies¹⁶ for horizontal starch-gel electrophoresis were applied. Partially hydrolyzed starch was purchased* and the starch gel prepared with 0.05 M tris(hydroxymethyl)aminomethane buffer pH 8.7. The electrodes were separated from the starch strips by a baffle system, filled with 0.25 M Tris buffer of the same pH and bridged with filter paper strips soaked in buffer. The samples were run for 6 hours at room temperature (23° C.) in a voltage gradient of 3 V/cm. After the run the 6-mm.-thick starch strips were cut longitudinally into 4 slices; the inner 2 slices were marked and placed in a solution of 30 mg. of sodium alpha-naphthyl acid phosphate and 300 mg. of Fast Blue BB† in 100 ml. of 0.1 M acetate buffer pH 4.6. The phosphatase bands were developed by overnight incubation in this mixture.

Culture and handling of Acetabularia. *Acetabularia mediterranea* and *Acicularia Schenckii* both were grown in Erdschreiber-Medium according to the

* From Connaught Laboratories, Toronto, Que., Canada.

† Both from Dajac Laboratories, Philadelphia, Pa.

methods described by Hämmerling¹⁷ and Beth.¹⁸ In culture and during the experiments the plants were illuminated with 2500 lux (fluorescence lamps) in 12-hour light-dark rhythms. The algae were dissected by means of fine scissors and grafts prepared by combining corresponding parts in telescopelike manner. After the operation the grafts were allowed to recover for 12 hours in diffuse light of low intensity.

Results

Both the species selected for transplantation experiments, namely *Acetabularia mediterranea* (*med*) and *Acicularia Schenckii* (*acic*) can be distinguished



FIGURE 1. Schematic illustration of the morphological traits of *Acetabularia mediterranea* (*med*) and *Acicularia Schenckii* (*acic*), both with full-grown caps.

morphologically by the structure of their caps; the rhizoids also differ characteristically in shape (FIGURE 1), a trait that is of great value for the identification of the components of certain multinucleate grafts to be described later.

When crude homogenates of either species were subjected to starch-gel electrophoresis all the acid phosphatase, as detected histochemically, was found to be localized in one band. The *acic* and *med* phosphatase differed from each other, however, in that the mobility of the *med*-type enzyme was significantly greater than the mobility of the *acic*-type enzyme. The species-specific mobilities of both phosphatase types are retained in a mixture of corresponding homogenates (FIGURE 2). The width as well as the intensity of both the bands appear about equal when comparable preparations are examined.

During early stages of normal development of the algae (10 to 30 mm. long)

acid phosphatase activity increases at a steady rate almost parallel to the increase in the protein content. In later stages, when caps begin to form, both acid phosphatase activity and total protein rise at a higher rate (FIGURE 3). During the period of growth and morphogenesis no change could be observed in the electrophoretic mobilities of either type of acid phosphatase.

Acid phosphatase synthesis continues in stalks of 30 mm.-long algae for approximately 1 week after enucleation. The enzyme activity rises to more than twice the original value during this period (FIGURE 4). Experimental data support the tacitly made assumption that in *Acetabularia* an increase in phosphatase activity reflects a corresponding net synthesis of enzyme protein. The already described parallel behavior of enzyme activity and the total content of protein during the normal development of *Acetabularia* suggests a net

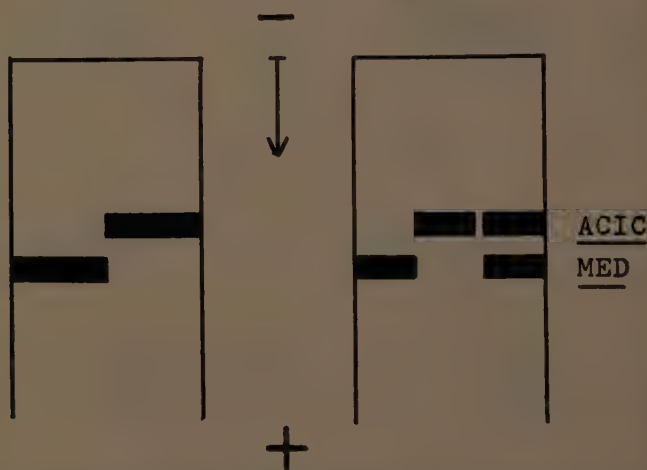


FIGURE 2. Diagrammatic representation of zymograms demonstrating the difference in the electrophoretic mobility of *med*-type and *acic*-type phosphatase (*left*) and the retention of species-specific mobilities in a mixture of both homogenates when compared to the individual components (*right*).

synthesis of enzyme protein; the observation that in the dark both enzyme activity and protein content remain essentially constant also support the idea that enzyme activity reflects net synthesis of an enzyme protein. It could be argued, however, that the enzyme protein synthesized in the absence of the nucleus, although functional, may have suffered some structural changes. In order to test this possibility, *med* and *acic* plants of the proper size were rhizoid-amputated, and the remaining stalks were examined for phosphatase mobilities after various periods of time extending up to 25 days following the enucleation. In all the material examined only the species-characteristic phosphatase types could be detected as represented by bands of normal width and position (FIGURE 5). These results indicate that the anucleate cytoplasm is capable of synthesizing not only functionally normal proteins but also structurally normal ones, since it has been reported that even minute differences in the structure of certain proteins result in significant alterations in electrophoretic mobil-

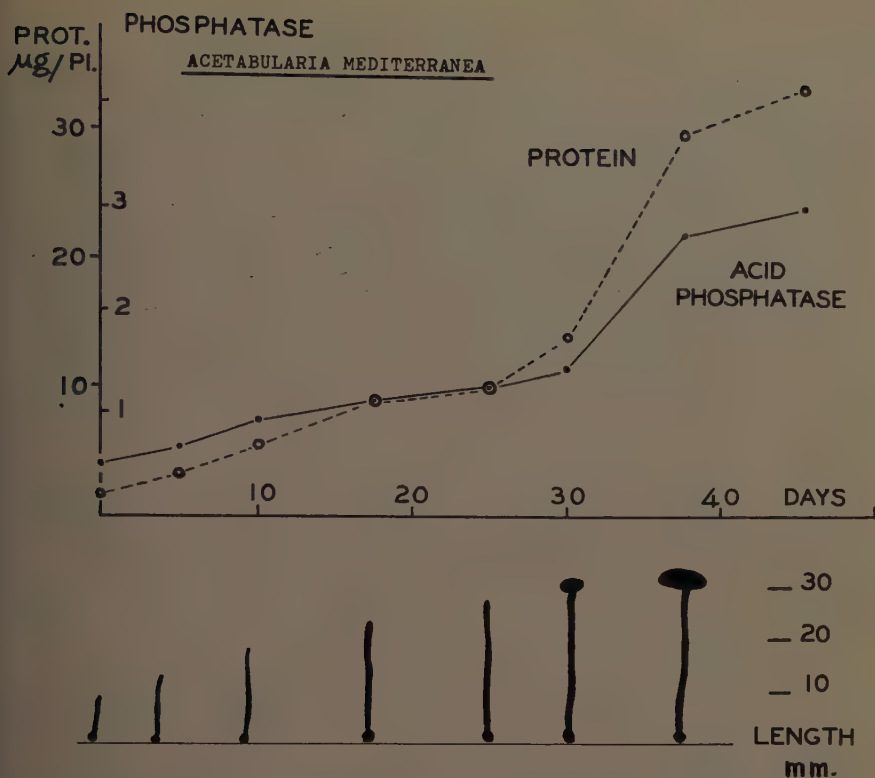


FIGURE 3. Increase in acid phosphatase activity and in protein content of *Acetabularia mediterranea* during normal development.

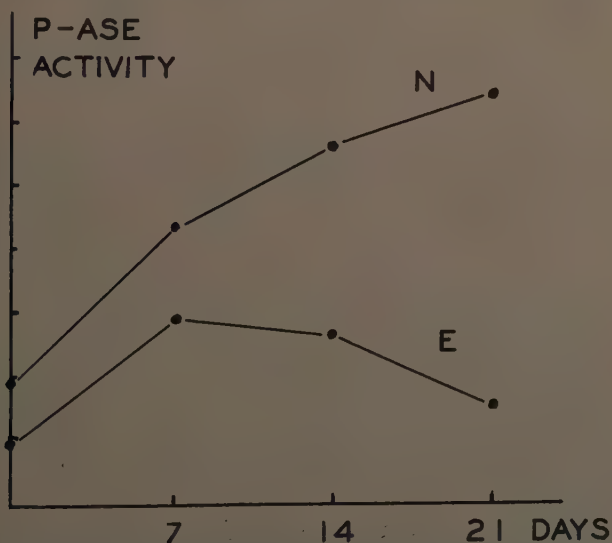


FIGURE 4. Changes in the activity of acid phosphatase in rhizoid amputated 30 mm. long *med* plants (E) as compared to nucleate controls (N). Enucleation was carried out at time zero.

ity.¹⁹⁻²¹ The observed stability of each phosphatase type in corresponding anucleate plants not only bears on the capacities of the cytoplasm *per se*, but also serves as a background against which *de novo* activities of an implanted species-foreign nucleus could be detected.

In *Acetabularia* interspecific nuclear transplantation is carried out by grafting the nucleus containing rhizoid of one species onto an enucleated stalk of another species. Inherent to this procedure, nuclear transplantation is always accompanied by the transfer of a small amount of cytoplasm that is carried over with the rhizoid into the recipient plant. Generally the stalks only of such grafts were examined in starch-gel electrophoresis. The remaining rhi-

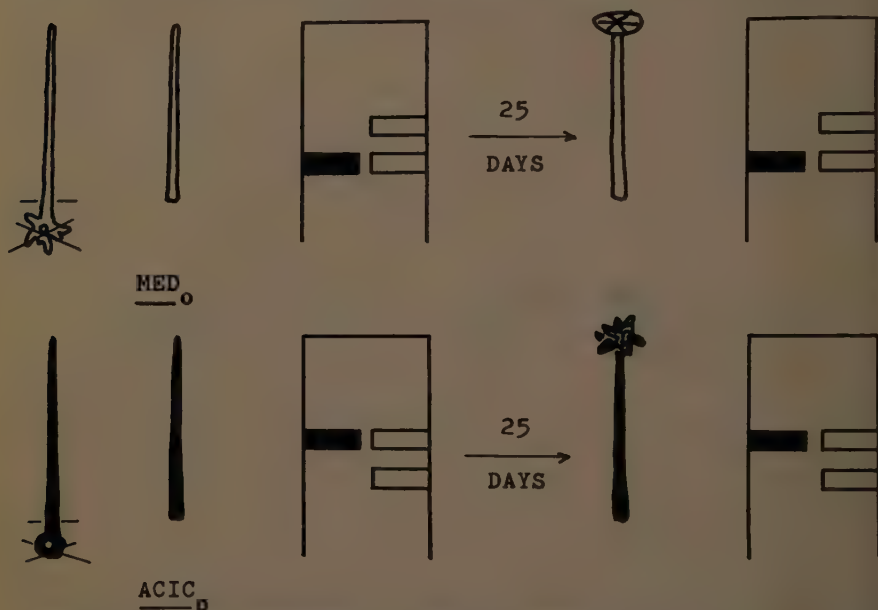


FIGURE 5. Diagram illustrating the stability in enucleated algae of both types of phosphatase (bands filled in with black). The outlined bands to the right indicate the position of bands which were obtained from a mixture of *med* and *acic* homogenates, which was included in every run to serve as reference.

zoids were collected and tested for their regenerative capacities. Successful regeneration of a new stalk confirmed the original presence in the rhizoid of a nucleus and its survival in the graft.

In the first series of experiments *med* nuclei were transplanted to *acic* stalks yielding *acic-med*₁ interspecific grafts. One group of such grafts was tested on the following day, another group cultured for 15 days and was then tested. One-day-old grafts contained the original *acid*-type phosphatase as the only recognizable component. No trace of an *acic*-type enzyme was detected in zymograms of 15-day-old grafts; instead the *med*-type phosphatase was observed as the only component (FIGURE 6). The synthesis in these grafts of an enzyme of the nucleus donor type is not difficult to understand; rather surprising, however, is the complete disappearance of the enzyme type originally present in

the cytoplasm. The absence of its own nucleus could not have caused this effect, since we already have seen that in anucleate *acic* plants the *acic*-type phosphatase can be detected in undiminished intensity as late as 25 days after the enucleation.

The observed effect may be explained in several ways. First, insertion of the species-foreign nucleus may have caused inactivation or even degradation of the pre-existing enzyme in addition to an independent net synthesis of *med*-type phosphatase. One could also imagine that, after enucleation, the level of phosphatase activity is the result of two processes: a rapid, spontaneous decay, compensated by a synthesis of new molecules. An implanted foreign nucleus could conceivably take over the synthetic sites of the cytoplasm, hence the prevailing process of degradation would result in the loss of the original

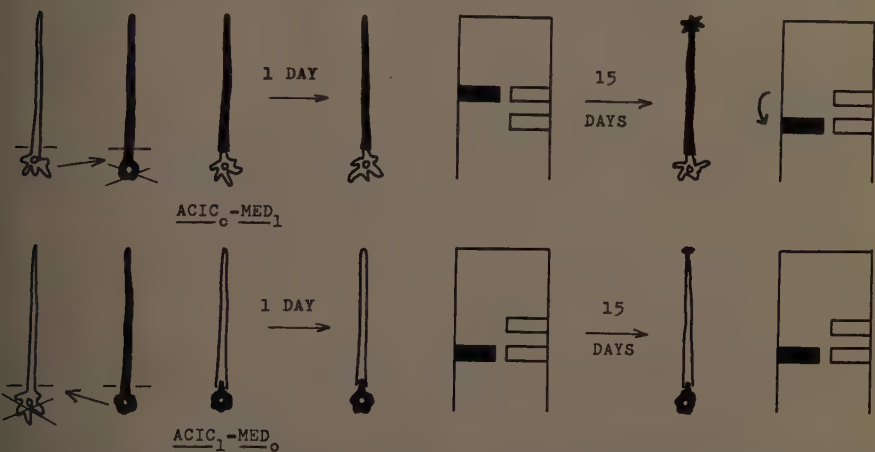


FIGURE 6. Diagrammatic representation of nuclear transplantation experiments. Top row: $\underline{acic}_0 - \underline{med}_1$. Bottom row: $\underline{acic}_1 - \underline{med}_0$.

enzyme type. As a third alternative, one could envision a direct conversion on the molecular level of one enzyme type into the other type.

It was interesting to find that an analogous conversion of enzyme types did not occur in reciprocal grafts obtained by transplanting *acic* rhizoids to *med* stalks ($\underline{acic}_1 - \underline{med}_0$). After 1 day, and also after 25 days, only the *med*-type enzyme was detected in the stalks of such grafts (FIGURE 6).

Interspecific grafts of the first described type, viz. $\underline{acic}_0 - \underline{med}_1$ were reinvestigated in greater detail in the hope of finding a clue as to which of the above considered mechanisms might be responsible for the observed effects. $\underline{Acic}_0 - \underline{med}_1$ grafts were examined at one-day intervals after rhizoid implantation. Individual stalks and homogenates from several stalks both were tested. The *acic*-type phosphatase originally present in the stalks is maintained as sole component for two days following the operation. On the third day the *med*-type phosphatase appears in the form of a faint additional band. About the fourth day the *med* band has gained in intensity so that both bands appear equally dark. The *acic* band is reduced in intensity on the fifth day and cannot be detected after the sixth day (FIGURE 7).

Occasionally, in preparations from 3- to 5-day-old grafts, a band was observed that did not correspond to a *med* or an *acic* band with respect to its position in the starch strips. The new band was located halfway between the two reference bands and was significantly wider than either of the two bands (FIGURE 7).

In view of these findings let us reconsider the previously suggested mechanisms. An inactivation or degradation of the cytoplasmic (*acic*-type) phosphatase, as initiated by the foreign nucleus, would require rather specific agents, since it would depend on a discrimination between both enzyme types. This seems highly unlikely. The second possibility—inhibition of *acic* enzyme

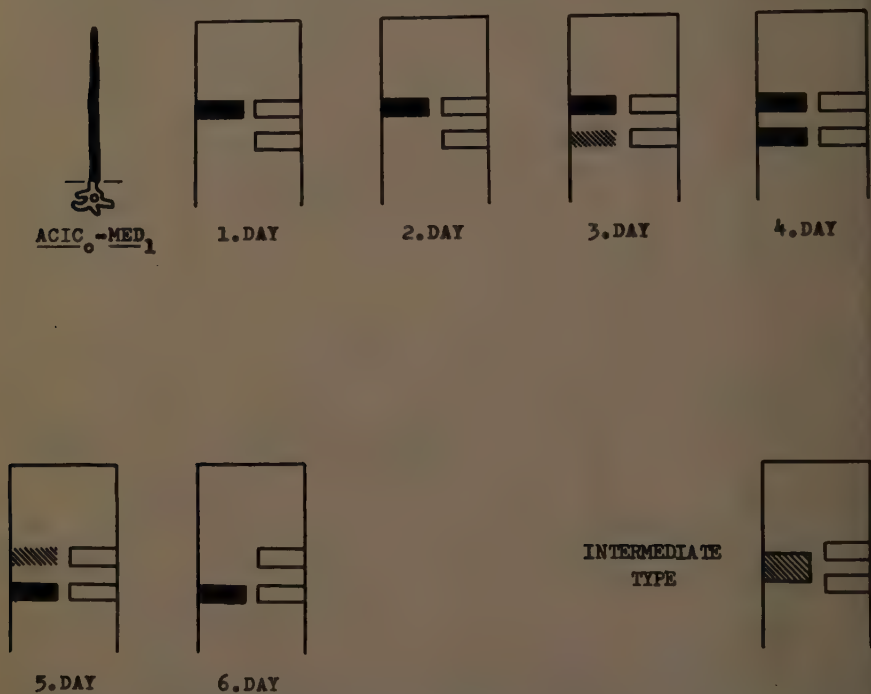


FIGURE 7. Schematic illustration of the change in enzyme types as a result of nuclear transplantation ($\underline{acic}_0 - \underline{med}_1$) when analyzed at one-day intervals.

synthesis and the disappearance of this enzyme as a consequence of a high rate of spontaneous decay—can be dismissed on the grounds that in *Acetabularia* the phosphatase activity remains fairly constant under conditions where protein synthesis is strongly impaired. On the basis of the experimental data, the third postulated mechanism is the most likely, namely a conversion on the molecular level of one enzyme type into the other. Not only does the timely coincidence of the two processes—appearance of the *med* enzyme and disappearance of the *acic* enzyme—point in this direction. The existence in certain grafts of a stage during which all acid phosphatase activity is localized in a new, intermediate-type band also supports this view.

The apparent incapacity of an *acic* nucleus to change to its specifications the phosphatase type after transplantation to a *med* cytoplasm needed further

investigation. It was felt that the *med* cytoplasm might be lacking factors necessary for the foreign nucleus to express its genetic information. Hence a combination was examined of a *med* and an *acic* nucleus in a common hybrid cytoplasm of a stalk which had been regenerated under the control of both nuclei (*acic*₁-*med*₁). The electrophoretic analysis revealed that also in *acic*₁-*med*₁ plants only the *med*-type enzyme was synthesized (FIGURE 8). The same enzyme type was found in stalks that had been dissected from the binucleate plants and maintained in culture for 15 days. Although the *acic* nucleus did not contribute to the hybrid cytoplasm any information with respect to the phosphatase type synthesized, other activities of this nucleus became quite

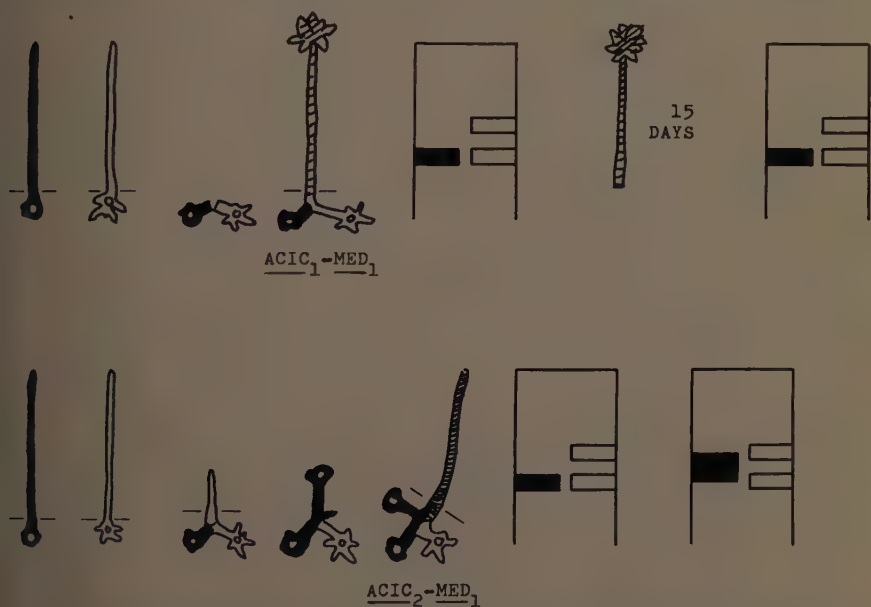


FIGURE 8. Schematic representation of the microsurgical operations necessary for the preparation of *acic*₁-*med*₁ binucleate, and *acic*₂-*med*₁ trinucleate algae, as well as representation of the phosphatase types found in the stalks of these multinucleate grafts.

obvious. For example, the caps grown by binucleate plants were very similar in their form to pure *acic* caps.

When an additional *acic* nucleus is grafted to the *acic*₁-*med*₁ system, a trinucleate *acic*₂-*med*₁ plant is obtained. This nuclear combination gave ambiguous results. In some of the trinucleate plants only the *med*-type phosphatase was found, in others a characteristically wide, intermediate band could be observed in the starch strips, which appeared to be identical to the one observed after nuclear transplantation (FIGURE 8). Here, however, the new enzyme type seemed to be a stable component rather than a transient type of short duration, as found during the conversion of phosphatase types.

In another experiment a number of fully regenerated binucleate *acic*₁-*med*₁ algae were set aside and the *med*-nucleus removed by amputation of the corresponding rhizoid (FIGURE 9). The algae thus obtained consisted of an *acic*

nucleus combined with a hybrid cytoplasm. The possibility was to be tested whether an *acic* nucleus was able to induce the formation of its own enzyme type in a cytoplasm of mixed composition. Algae prepared in this manner were kept in culture for 15 days; the stalks were then removed and tested. The negative results of this experiment—persistence of the *med*-type enzyme—indicated that the presence of a *med* component *per se* in the cytoplasm was sufficient to cancel out any possible influence on the enzyme type by the *acic* nucleus.

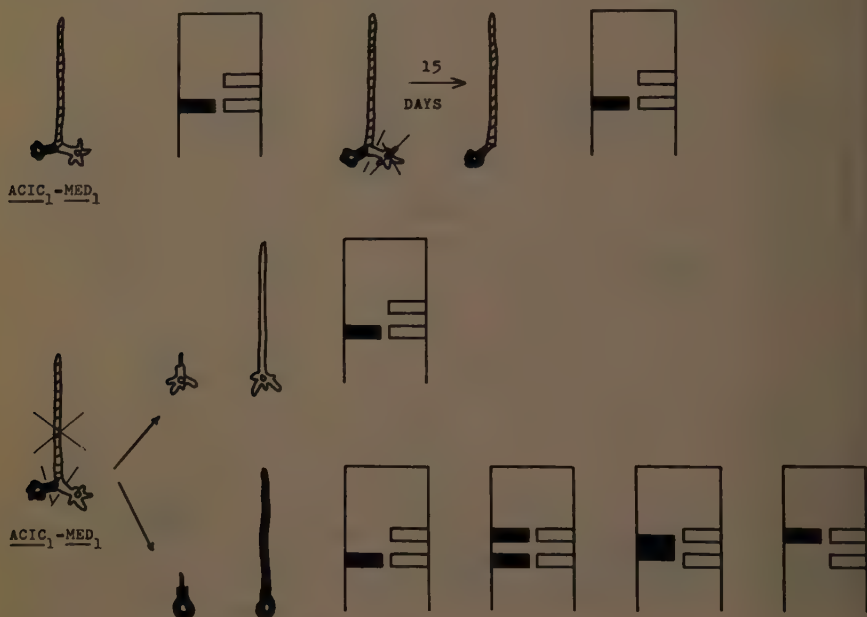


FIGURE 9. Top row: experimental procedures applied to test the capacity of an *acic* nucleus to change the phosphatase type in a hybrid cytoplasm. Bottom row: schematic illustration of an experiment testing the persistency of *med* traits in a hybrid cytoplasm by separate regeneration of the *med* and *acic* rhizoids from an *acic*₁-*med*₁ binucleate plant.

The unlimited capacity of rhizoids to regenerate a whole plant offered the possibility of examining the degree of persistency of the *med* component in the cytoplasm. Again *acic*₁-*med*₁ binucleate plants were prepared; after full regeneration the *acic* and *med* rhizoids were removed by dissection and maintained in culture. The *acic* rhizoids gave rise to plants which could not be distinguished morphologically from normal *acic* plants. When examined in starch gel electrophoresis, however, the presence of a *med* trait was indicated by the frequent appearance of *med*-type phosphatase bands or intermediate-type bands in the starch strips (FIGURE 9). This is even more remarkable since only a very small quantity of hybrid cytoplasm was originally present in the rhizoids, which had been greatly diluted during the ensuing process of regeneration by presumably pure *acic* cytoplasm. The *med* traits disappeared, however, when the rhizoids from such algae were subjected to a second regeneration.

Conclusions

The data presented in this paper strongly imply that in *Acetabularia* a pre-existing species-specific enzyme protein can be structurally modified by transplanted nuclear or cytoplasmic factors from another species. The process of modification is probably genetically controlled since the protein product seems to be identical to the corresponding enzyme protein of the inducer species.

The finding of a wide, intermediate type phosphatase band supports the postulated mechanism of molecular conversion and suggests that a heterogeneous population of intermediate-type phosphatase molecules has arisen: the excessive width of the band being due to the microheterogeneity within the population, and the intermediate position of the band being due to a major step in the conversion.

Both species investigated differ in their capacity to determine the phosphatase type in interspecific combinations. Any *med* component—nucleus or cytoplasm—present in an interspecific graft will invariably dominate over the *acic* component in that only *med*-type enzyme is produced. One might wonder whether the “dominant” *med*-type enzyme is not a more advanced stage of the *acic*-type, particularly if one assumes that both types of molecules have in common a basic structural design. On this hypothesis the *acic*-type enzyme would be a natural precursor of the *med* enzyme, even in *med* plants. The intermediate-type protein, as found in certain interspecific grafts, probably represents one of the last steps in the biosynthesis; the intermediate form need not necessarily be detectable in normal *med* plants since under normal conditions very little protein will be in transient stages at any given moment.

The capacity to induce or maintain the *med*-type phosphatase remains effective even after great dilution of the cytoplasm which carries this factor. Eventually, however, the factor is lost after repeated regeneration of such plants. Whether or not the responsible units undergo a replication in the host cytoplasm is difficult to decide. At any rate, the tenacious retention of such factors in the cytoplasm confirms previous findings of high synthetic capacities of the anucleate cytoplasm.

Acknowledgment

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References

1. BRACHET, J., H. CHANTRENNE & F. VANDERHAEGE. 1955. Recherches sur les interactions biochimiques entre le noyau et le cytoplasme chez les organismes unicellulaires. II. *Acetabularia mediterranea*. Biochim. et Biophys. Acta. **18**: 544.
2. STICH, H. 1953. Der Nachweis und das Verhalten von Metaphosphaten in normalen, verdunkelten und Trypaflavin-behandelten Acetabularien. Z. Naturforsch. **8b**: 36.
3. STICH, H. 1955. Synthese und Abbau der Polyphosphate von *Acetabularia* nach autoradiographischen Untersuchungen des P^{32} -Stoffwechsels. Z. Naturforsch. **10b**: 281.
4. VANDERHAEGE, F. 1954. Les effets de l'énucléation sur la synthèse des protéines chez *Acetabularia mediterranea*. Biochim. et Biophys. Acta. **15**: 281.
5. WERZ, G. 1955. Unpublished data, cited in: Hämmerling. J. Biol. Zentralbl. **74**: 545.
6. CLAUSS, H. 1958. Ueber quantitative Veränderungen der Chloroplasten- und cytoplasmatischen Proteine in kernlosen Teilen von *Acetabularia mediterranea*. Planta. **52**: 334.

7. BALTUS, E. 1955. Influence du noyau sur le maintien de l'aldolase dans le cytoplasme. Congr. intern. Biochem. Résumés communs 3^e Congr. Brussels. : 76.
8. KECK, K. & H. CLAUSS. 1958. Nuclear control of enzyme synthesis in *Acetabularia*. Bot. Gazette. **120**: 43.
9. CLAUSS, H. 1959. Das Verhalten der Phosphorylase in kernhaltigen und kernlosen Teilen von *Acetabularia mediterranea*. Planta. **52**: 534.
10. MAZIA, D. 1952. Physiology of the cell nucleus. In Modern Trends in Physiology and Biochemistry. : 77. E. S. G. Barron, Ed. Academic Press. New York, N. Y.
11. HUNTER, R. L. & C. L. MARKERT. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science. **125**: 1294.
12. DIXON, G. H. & O. SMITHIES. 1957. Zone electrophoresis of cabbage enzymes in starch gels. Biochim. et Biophys. Acta. **23**: 198.
13. MARKERT, C. L. & F. MØLLER. 1959. Multiple forms of enzymes: Tissue, ontogenetic, and species-specific patterns. Proc. Natl. Acad. Sci. **45**: 753.
14. KECK, K. 1960. Nucleo-cytoplasmic interactions in the synthesis of species-specific proteins in *Acetabularia*. Biochem. Biophys. Research Commun. **3**: 56.
15. BETH, K. 1943. Ein- und zweikernige Transplantate zwischen *Acetabularia mediterranea* und *Acicularia Schenckii*. Z. Vererbungslehre. **81**: 271.
16. SMITHIES, O. 1955. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. Biochem. J. **61**: 629.
17. HÄMMERLING, J. 1944. Zur Lebensweise, Fortpflanzung und Entwicklung verschiedener Dasycladaceen. Arch. Protistenk. **97**: 7.
18. BETH, K. 1953. Experimentelle Untersuchungen über die Wirkung des Lichtes auf die Formbildung von kernhaltigen und kernlosen *Acetabularia*-Zellen. Z. Naturforsch. **8b**: 334.
19. MARKERT, C. L. & E. APPELLA. 1961. Ann. N. Y. Acad. Sci. **94** (3): 678.
20. HUNT, J. A. & V. M. INGRAM. 1958. The chemical effects of gene mutations in some abnormal human haemoglobins. In Symposium on Protein Structure. : 148. A. Neu-berger, Ed. Wiley. New York, N.Y.
21. PAULING, L., H. A. ITANO, S. J. SINGER & I. C. WELLS. 1949. Sick-cell anemia—a molecular disease. Science. **110**: 543.

GENETIC CONTROL OF THE ESTERASES IN THE PROTOZOAN *TETRAHYMENA PYRIFORMIS**

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Two years ago Markert and Møller (1959) introduced the concept of an *isozyme*, which they defined as one of the different molecular forms of an enzyme. They proposed that each isozyme could be controlled by a separate gene or that an isozymic series could be controlled by a single gene. Essentially the problem centers on the way in which genes control a protein: how a protein is made and how it is modified in structure and function under the influence of genotypic and microecological factors. According to the present genetic dogma the deoxyribose nucleic acid (DNA) code is translated into a ribose nucleic acid (RNA) code which specifies the sequence of amino acids in a polypeptide (see Watson, 1960; Ingram, 1960; Spiegelman, 1960). These sequences automatically determine the secondary and tertiary structures of a protein. Analysis of the genetic control of an isozyme would, therefore, further our understanding of these relationships.

Tetrahymena pyriformis possesses several different esterases, some of which form an isozymic series (Allen, 1960a). Although this study suggested that the series might be under the control of a single locus, only recently has a definitive answer been obtained. This report will present these observations and also evidence for the genetic control of other members of the family of esterases. The behavior of the isozymes in heterozygotes suggests that they are also under epigenetic control (Nanney, 1958) and that the pattern of nuclear differentiation and vegetative assortment is probably similar to that observed for other loci in this organism.

Materials and Methods

A detailed description of the materials and some of the methods employed in this study was given in an earlier report (Allen, 1960a). Representatives of four of the inbred strains, A, B, C and D, of variety 1 of *T. pyriformis* were sampled for their esterases. In the present study two of these strains, B and C, served as the source of the parental cultures used in making crosses. They are now in the 11th and 10th generations of inbreeding, respectively. The origins of these strains were described by Nanney (1959).

All crosses were made from cultures grown on 1 per cent proteose-peptone at 30° C. using sterile technique. The crosses were made in sterile glass-distilled water, and the mating pairs were separately isolated into depression slides containing peptone. In some crosses the exconjugants were separated. The culture that developed was tubed up in peptone, and after the plateau phase of growth had been reached, it was transferred to 250 ml. Erlenmeyer flasks with 100 ml. of peptone, adjusted to pH 7.0. Extracts were made after 5 days of growth (plateau) at 30° C. However, before any culture was used,

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the pair giving rise to it was carefully screened for immaturity, a sign that conjugation has been completed in the pair. Only immature cultures were used.

Crude extracts of concentrated cells from 100 ml. of culture were prepared by repeated freeze-thawing. In a few experiments preparations of cilia from cultures of strains B and C were made according to the method outlined by Preer and Preer (1959, p. 90). The cilia preparations were also frozen-thawed.

The electrophoretic separations were carried out in starch gels using a borate buffer system at pH 7.5 except in a few experiments in which a pH of 8.5 or 9.5 was used. The application of the zymogram technique (Hunter and Markert, 1957) to *T. pyriformis* was described earlier (Allen, 1960a). For histochemical determinations α -naphthyl acetate, β -naphthyl acetate, α -naphthyl propionate, and α -naphthyl butyrate were used as substrates, and the stable diazotate of 4'-amino-2',5'-dimethoxybenzanilide (Fast Blue RR)* was used as dye-coupler. In some experiments starch strips were preincubated in eserine sulfate, at a final concentration of 10^{-4} M. The substrates, α -naphthyl propionate and α -naphthyl butyrate, only, were used for the identification of the E-1 and E-2 esterases in the progeny of crosses. For facilitating the identification of the E-1 esterases one of the starch strips was exposed to a reaction mixture containing α -naphthyl propionate, Fast Blue RR, and sodium taurocholate, at a final concentration of 10^{-2} M. After 3 to 4 hours of incubation in the reaction mixtures, the starch strips were removed and photographed with a Polaroid Land camera. In a few experiments the intensity of staining of the E-1 esterases was measured directly from starch strips (using starch poured 1 hour before use) with a modified Photovolt Densitometer.†

The localization studies were performed on air-dried preparations on clean slides. No fixatives and no egg albumin were used, since they were found to destroy the patterns of localization. The same histochemical reagents used above were also used here, except that the stable diazotate of o-dianisidine (Fast Blue BN)‡ was used as dye-coupler. The slides were incubated in the reaction mixtures for varying lengths of time, but the optimum incubation time was 35 min. The slides then were rinsed in distilled water and mounted in 1 per cent glycerine jelly.

The Esterases of Variety 1 T. pyriformis

The proteins of variety 1 of *T. pyriformis* can be resolved into a number of zones having esterase activity (FIGURE 1). These zones can be separated into two classes on the basis of substrate specificity and reaction to eserine sulfate and sodium taurocholate. At a pH of 7.5 during the electrophoretic separation the class I esterases include two groups of isozymes: the B group with four to five isozymes, which migrates towards the cathode, and the C group with four isozymes, which migrates towards the anode. At pH 8.5 and 9.5 each group contains a pair of isozymes (FIGURE 2). The B group is found in several inbred strains (A, B, and D) while the C group is limited to strain C (FIGURE 3). The class I esterases are inhibited by 10^{-4} M eserine sulfate and activated

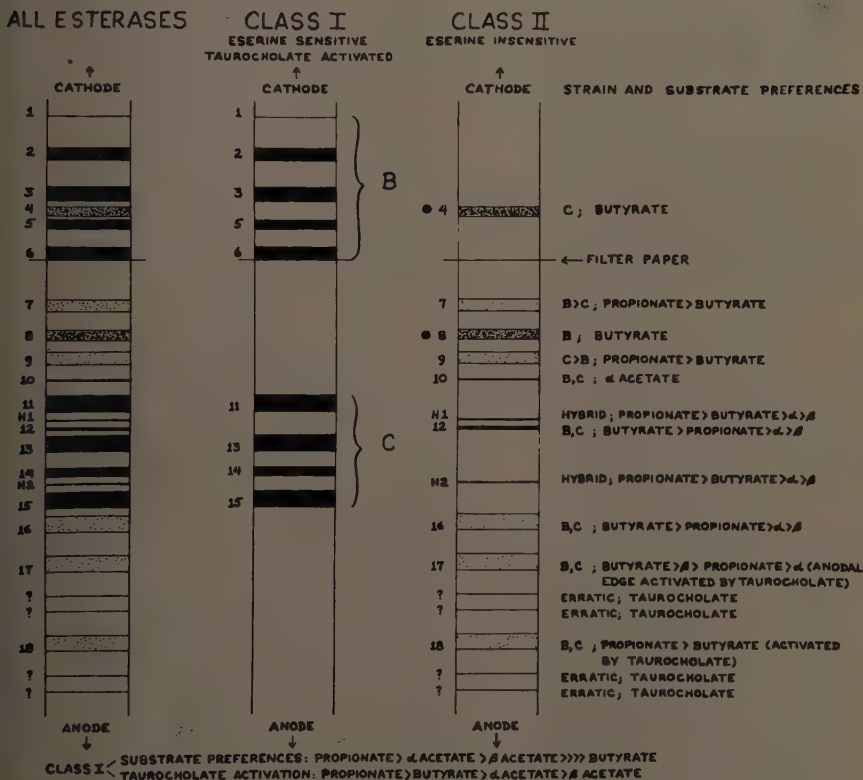
* General Dyestuff Company, New York, N.Y.

† Model 525, modified by Robert L. Hunter in cooperation with the Photovolt Corporation, New York, N.Y.

‡ General Dyestuff Company, New York, N.Y.

by $10^{-2} M$ sodium taurocholate (FIGURE 4). They split α -naphthyl propionate, α -naphthyl acetate and β -naphthyl acetate, in order of efficiency, but split α -naphthyl butyrate very poorly, if at all (FIGURE 5). Visual inspection shows that the activation by taurocholate depends upon the substrate present. The order of activation was the following: propionate \gg butyrate $>$ α -acetate $>$ β -acetate. The class I esterases will be referred to hereafter as the E-1 esterases.

CHARACTERIZATION OF ESTERASES IN *T. PYRIFORMIS*, VARIETY 1



The class II esterases are a family of different enzymes, whose substrate preferences are indicated on the right of FIGURE 1. Two of these esterases at zones 4 and 8 only split α -naphthyl butyrate. They show strain differences, the esterase at zone 8 being found in strain B and the esterase at zone 4 being found in strain C. Electrophoretic separation at pH 8.5 shows little change in position over separation at pH 7.5 (FIGURE 6). Hereafter, these two esterases will be referred to as the E-2 esterases.

The E-1 esterases are of considerable interest since they are made up of an isozymic series. An earlier report (Allen, 1960a) showed that all members of

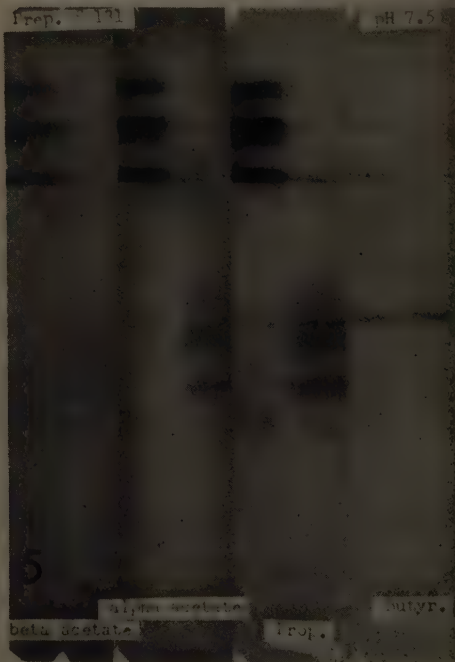
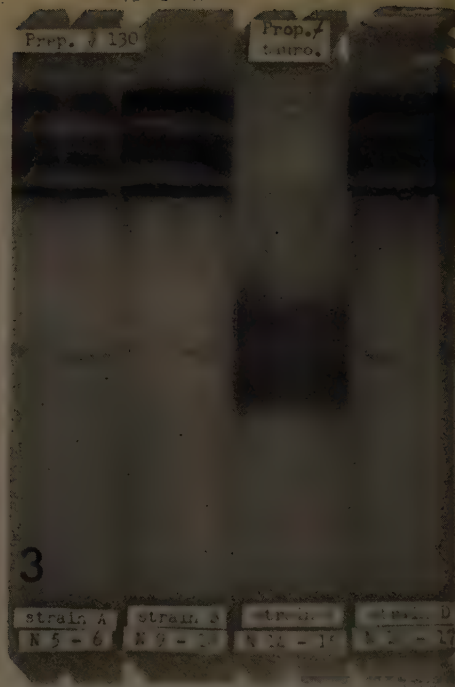
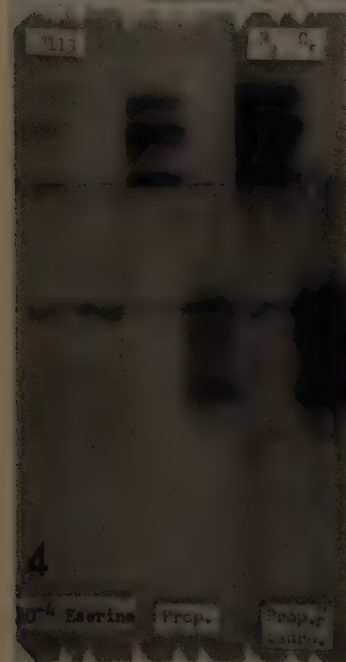
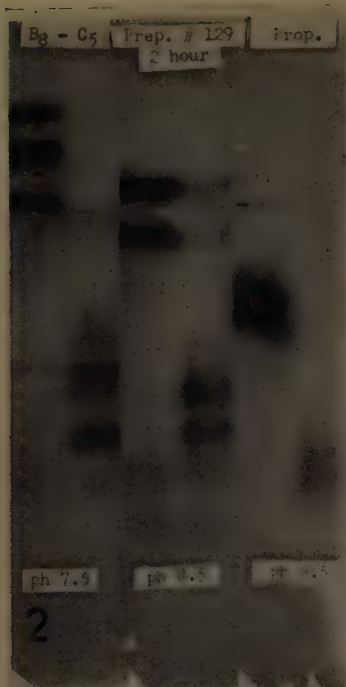


FIGURE 2. Effect of pH on migration of E-1 esterases (B on left, C preparation on right of same starch columns). Substrate: α -naphthyl propionate. Incubated 2 hours.

FIGURE 3. B and C groups of E-1 esterases in four inbred strains. Substrate: α -naphthyl propionate with sodium taurocholate. Incubated 4 hours.

FIGURE 4. Inhibition and activation of E-1 esterases (B on left, C on right) with α -naphthyl propionate as substrate. Incubated 3 hours.

FIGURE 5. Substrate specificities of E-1 esterases (B on left, C on right). Incubated 4 hours.

an isozymic group are present in a single cell as determined by clonal analysis. Also, all members are present under a variety of growth conditions, but they differ in their intensity of activity. If cells are starved preparatory for mating, the activities of the anodal isozymes in each group are enhanced. If cells are grown at 16° C. for one to three weeks, then the activities of the cathodal isozymes in each group are enhanced. This pattern is also observed in the heterozygote (which contains both groups) and occurs in *each* group of isozymes. If cultures are grown for progressively longer periods of time before extractions are made, a complex sequence of activities is observed in each group as a function of the growth cycle.

Preliminary studies suggest that isozymic activities may also vary in different parts of the cell, although some difficulty in precise definition may be anticipated depending upon the degree of solubility of these esterases. Cilia preparations have a different pattern of activities than extracts of the whole cell (FIGURE 7). Although the difference is only quantitative in these preparations, one isozyme in each group appears to be greatly enhanced. In a closely related ciliate (*Tetrahymena geleii* S), Seaman (1951) demonstrated a specific acetylcholinesterase associated with the fibrillar system of the pellicle. Thus cell fractionation procedures may prove very useful in defining the different isozymes. With further refinement of these techniques and extension of this type of analysis to include many more cell fractions it may even be possible to achieve qualitative differentiation of the isozymes.

An attempt to find different patterns of intracellular localization using whole cells will not be fruitful in view of the failure to differentiate the esterases as to class or strain, using the localization techniques (Allen, 1958). However, striking but similar differences in pattern were observed in the *total* esterase picture of all strains during the course of the growth cycle. These observations may be pertinent in the present context, since they do demonstrate significant shifts in site of activity within the cell. During the logarithmic phase of the growth cycle the localizations are confined to discrete "vesicles" in a perinuclear position (FIGURE 8) and, in dividing cells, the perinuclear orientation is retained. The "cortex" is poorly developed and lacks activity. The activity per cell is uniform within the cell population. When cells reach plateau the vesicles extend from the perinuclear position to the posterior of the cell (FIGURE 9). The cortex is fully developed, very active, and has a characteristic architecture: from indentations in the cell membrane run "fibrillae" (these may represent overlapping layers of cilia in a flattened preparation); they occur at regular intervals perpendicular to the cell surface. Below these indentations in the cell membrane, which is active enzymatically, are granules that are also active. If focussed on the surface of the cell, the pattern of activities is similar to silver-line preparations, which outline the kinetal system. Dipping the slides in ethanol removes the cortical structure entirely. During the stationary period the most active cells show a well-developed cortex, and the vesicles fill the cytoplasm of the cell (FIGURE 10). The cell population, however, shows extreme variability both in cell size and activity, and these are correlated.

These changes represent a complex of the activities of many different esterases, yet they demonstrate that certain sites within the cell are esterase-positive and

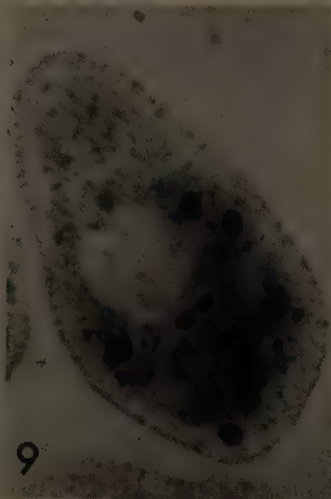
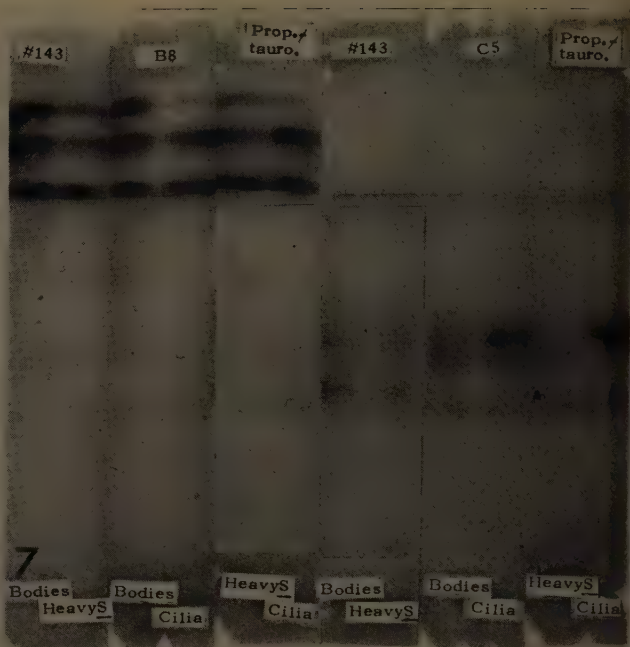
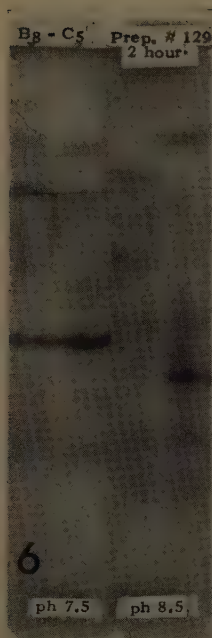


FIGURE 6. Effect of pH on migration of E-2 esterases (B on left, C on right). Substrate: α -naphthyl butyrate. Incubated 2 hours.

FIGURE 7. Comparison of cilia fractions with body fractions (B set on left, C set on right). Compact pellet is labeled "bodies"; flocculent precipitate immediately above compact pellet is labeled "Heavy S"; milky supernatant above is labeled "Cilia." Substrate: α -naphthyl propionate with sodium taurocholate. Incubated 4 hours.

FIGURE 8. Intracellular localization of esterase activity during logarithmic phase of growth cycle. Substrate: α -naphthyl acetate. $\times 760$.

FIGURE 9. Intracellular localization of esterase activity during plateau phase of growth cycle. Substrate: α -naphthyl acetate. $\times 760$.

FIGURE 10. Intracellular localization of esterase activity during stationary phase of growth cycle. Substrate: α -naphthyl acetate. $\times 760$.

that their activities change depending upon whether the cells are actively dividing or are merely growing in size. From these observations and the preliminary studies of cell fractions, it seems that a microtopological approach might be very helpful towards understanding the nature of an isozyme. It might even be possible to localize each isozyme of a group to a different cell structure.

Genetic Studies

Before presenting the results of the genetic studies, the expectations of conjugation in *T. pyriformis* will be briefly reviewed (FIGURE 11). Tetrahymena possesses one macronucleus and one micronucleus. The macronucleus contains many chromosome sets organized into physiological units called subnuclei (Allen and Nanney, 1958) that control the vegetative phenotypes of the

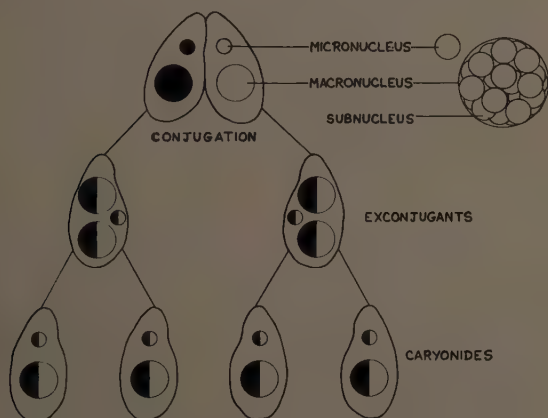


FIGURE 11. Diagram of conjugation in *T. pyriformis*.

cell. During conjugation the old macronucleus is discarded, and new ones form from the products of the zygotic nucleus. In each conjugant of a mating pair the micronucleus undergoes meiosis, giving rise to four haploid nuclei, three of which disintegrate. The remaining nucleus divides mitotically to give rise to the migratory and stationary pronuclei. Reciprocal fertilization results in a zygotic nucleus that is diploid and identical in genotype in the two exconjugants. Different pairs may differ in genotype. Thus, after a cross is made, each pair is isolated into a separate container. Genetic ratios are obtained from the distributions of phenotypes observed in the different pairs. For some characters differences in phenotype may be found within the pair (see Sonneborn, 1957): between exconjugants and even between the two daughter cells (or sister caryonides) of the same exconjugant. A difference in phenotype between exconjugants is associated with differences in cytoplasmic factors, whereas a difference in phenotype between sister caryonides is associated with nuclear differentiation.

The phenotypes of the E-1 esterases are a pair characteristic although, as will be shown below under the *E-1 Heterozygote*, the phenotype of the heterozygote

is probably determined at the level of the caryonide. The results of several types of crosses are presented in TABLE 1. Crosses within a strain ($B \times B$ or $C \times C$) give rise to pairs possessing only the group of isozymes characteristic of the strain. In genuine heterozygotes produced in a cross of $B \times C$ all eight or nine isozymes are present; that is, all the isozymes of each group, so that these heterozygotes may be classified as $E-1B + C$ in phenotype. Unlike the esterases of maize (Schwarz, 1960) no hybrid molecules are formed.

Some exceptional pairs will be noted in the F1 generation. These are homozygotes: for the E-1 esterases, but also for characters at four different loci, some of which are unlinked (Allen, 1960*b* and unpublished). They arise as a result of genomic exclusion during conjugation, with members of the C strain varying in their capacity to produce this effect. As a matter of fact, the first crosses made in the genetic analysis of the esterases (Allen, 1960*a*) involved a member of the C strain, which produces almost 100 per cent transmission of all genes

TABLE 1
BREEDING BEHAVIOR OF E-1 ESTERASES

Cross	No. pairs tested	Esterases		
		$B + C$	B	C
$B \times B$	10	0	10	0
$C \times C$	22	0	0	22
$B \times C$	28	22	3*	3*
	22	8	14†	0
$B/C \times B/C$	177	88	45	44
$B/C \times B/B$	59	33‡	26	0
$B/C \times C/C$	40	24	0	16

* Homozygous at *E-1*, *E-2*, *mt* and probably *H* locus.

† The 14 "B" pairs were probably homozygous for B alleles at *E-1*, *E-2*, and *mt* loci.

‡ Two pairs, phenotypically "C" tested as heterozygotes.

from the other parent in the cross! The basis of this phenomenon is, at present, unknown, since cytological observation shows that C cells are present in the mating pairs and both exconjugants are recovered, yet conjugation appears to be normal in many more pairs than expected. Whatever its ultimate basis, this phenomenon has presented a major stumbling block in the analysis of all characters derived from the C strain.

Fortunately, some genuine heterozygotes are produced in crosses to the C strain. These heterozygotes breed normally, and good genetic ratios are obtained in the F2 and some backcross generations. In the F2 generation three classes of pairs were observed (FIGURE 12) in a 2:1:1 ratio—88 $B + C$: 45 B: 44 C. The backcross to strain B yields two classes of pairs (FIGURE 13) in a 1:1 ratio—33 $B + C$: 26 B, and the backcross to strain C yields two different classes of pairs (FIGURE 14) in an approximate 1:1 ratio—24 $B + C$: 16 C.

The results of testcrossing seven F2 segregants appear in TABLE 2. The two E-1B F2 segregants produced only B offspring, the two E-1C F2 segregants produced only C offspring, while the three E-1B + C F2 segregants produced all three classes of pairs in approximate 2:1:1 ratios. The exconjugants were

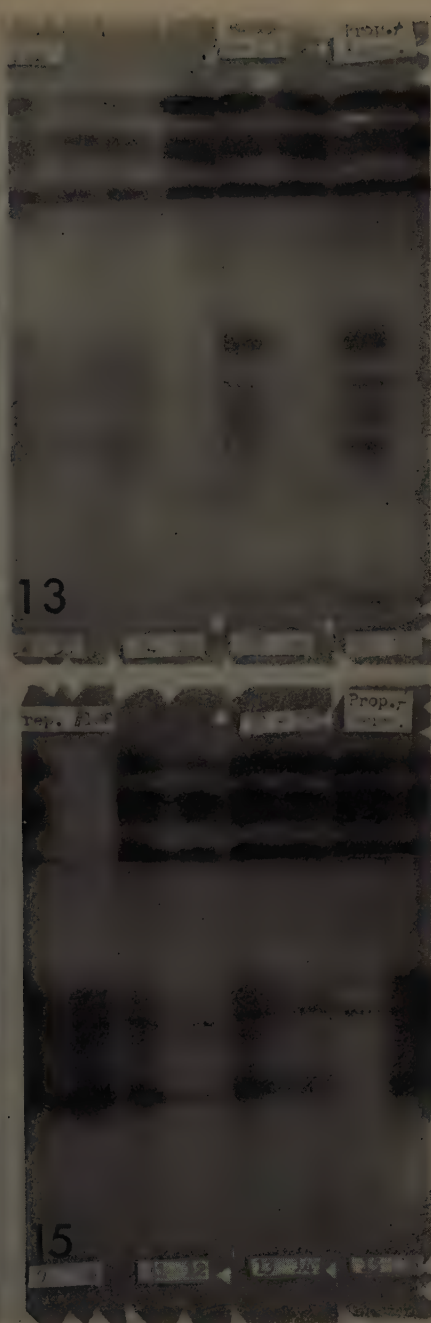
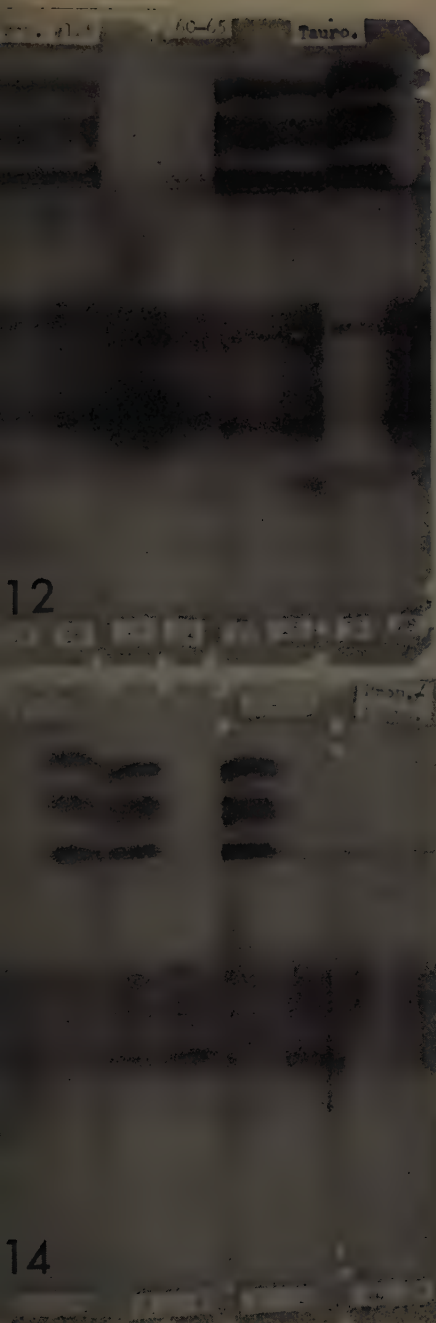


FIGURE 12. E-1 esterases of 8 F2 pairs. Substrate: α -naphthyl propionate with sodium taurocholate. Incubated 4 hours.

FIGURE 13. E-1 esterases of 8 backcross pairs (B/C \times B/B). Substrate: α -naphthyl propionate with sodium taurocholate. Incubated 4 hours.

FIGURE 14. E-1 esterases of 8 backcross pairs (B/C \times C/C). Substrate: α -naphthyl propionate with sodium taurocholate. Incubated 4 hours.

FIGURE 15. Eight subclones derived from an E-1B + C heterozygote. Substrate: α -naphthyl propionate with sodium taurocholate. Incubated 4 hours.

separated in the starred crosses and, except for the exconjugants of a heterozygous pair, the two were phenotypically alike. Exconjugants of heterozygotes varied quantitatively in their activities of the two groups of isozymes and occasionally qualitatively, that is, one of the exconjugants would possess activity for only one of the two groups of isozymes.

TABLE 2
TESTCROSSES OF F2 SEGREGANTS FOR E-1 ESTERASES

Phenotypes	No. pairs tested	Esterases		
		B + C	B	C
B	8*	0	8	0
B	5*	0	5	0
C	8*	0	0	8
C	8*	0	0	8
B + C	12*	5	5	2
B + C	8*	3	1	4
	21	10	4	7
	29	13	5	11
B + C	55	25	21	9

* Exconjugants separated.

TABLE 3
BREEDING BEHAVIOR OF E-2 ESTERASES

Cross	No. pairs tested	Esterases		
		B + C	B	C
B × B	10	0	10	0
C × C	22	0	0	22
B × C	28	22	3*	3*
	22	8	14†	0
B/C × B/C	39	20	11	8
B/C × B/B	40	18	22	0
B/C × C/C	40	26	0	14

* Homozygous at *E-1*, *E-2*, *mt* and, probably, *H* locus.

† The 14 "B" pairs were probably homozygous for B alleles at *E-1*, *E-2*, and *mt* loci.

The genetic data are consistent with the hypothesis that the two groups of isozymes are under the control of alleles at a single locus or at a cluster of closely linked loci. This locus (or region) will be designated as the *E-1* locus. The B strain is homozygous for the *E-1^B* allele, the C strain for the *E-1^C* allele. The heterozygote (*E-1^B/E-1^C*) has the *potentiality* of producing both groups of isozymes.

A second locus (*E-2*) governs the E-2 esterases, a conclusion reached from the results of the crosses shown in TABLE 3. The E-2B esterase is produced by

$E-2^B/E-2^B$ homozygotes, the $E-2C$ esterase by $E-2^C/E-2^C$ homozygotes. The heterozygote ($E-2^B/E-2^C$) produces both esterases ($E-2B + C$), but each esterase has about one half the activity of that of the homozygote. There is a suggestion that the $E-2$ heterozygote may also show variability in expression of the two esterases, but the pattern of variability is, as yet, not well documented.

Linkage tests of the $E-1$ and $E-2$ loci indicate independent assortment (TABLE 4). Both types of esterases were scored in the two backcross generations, and no significant deviation from the expected 1:1:1:1 ratio occurred. Linkage tests are in progress for the mating type (*mt*) locus and the $E-1$ and $E-2$ loci, but the data thus far are inconclusive. Tests have not, as yet, been initiated on the esterase loci and the H (serotype) locus, which is known not to be linked with the *mt* locus (Nanney, 1960b).

TABLE 4
INDEPENDENCE OF $E-1$ AND $E-2$ ESTERASES

Cross	$E-1B + C/E-2B + C$	$E-1B + C/E-2B$	$E-1B/E-2B + C$	$E-1B/E-2B$	Total
$B/C \times B/B$	11	11	7	11	40
	$E-1B + C/E-2B + C$	$E-1B + C/E-2C$	$E-1C/E-2B + C$	$E-1C/E-2C$	
$B/C \times C/C$	14	10	12	4	40

The E-1 Heterozygote

Although the $E-1$ heterozygote has the *potentiality* of producing both groups of isozymes, it varies in its expression of the two groups. Quantitative variation—and even qualitative variation—occurs between the exconjugants of a heterozygous pair. This variation is also observed within a *single* caryonide. When single cells were isolated from a caryonidal culture, the subclones showed considerable variability in their enzymatic activities (FIGURE 15), although the total activities of the four or eight isozymes present approached a common value. Some subclones had only B isozymes, some had only C isozymes, while others had some B and some C activity. The $E-1B$ or $E-1C$ subclones were stable, but the $E-1B + C$ subclones were metastable and gave rise to $E-1B + C$ and $E-1B$ and/or $E-1C$ cell lineages. Most of the cell lineages were $E-1B + C$, but the frequency of the three types varied with different $E-1B + C$ subclones.

The distribution of subclones from one $E-1B + C$ caryonide is depicted graphically (FIGURE 16). The graphs are based on Densitometer recordings. In general, the activity of a single isozyme was proportionate to the activity of a group, but in order to eliminate slight variations due to small differences in rates of cell division between cultures, which would influence the activity of a given isozyme, the group activities were calculated. The activities of each group were totaled, then the percentage of C group activity was determined for each subclone. Thus a subclone with only B activity would have a "0" per cent

value, while one with only C group activity would have a "100" per cent value. Those with some C group and some B group activities have intermediate values. Treatments of two kinds (starvation for one week in distilled water before isolating single cells into peptone; or growth of isolates at 16° C. for three weeks before transferring the cultures to flasks at 30° C.) had no effect on the distri-

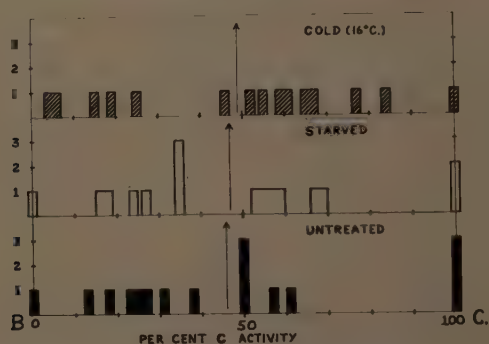


FIGURE 16. Lack of effect of two treatments on distribution of subclones derived from an E-1B + C heterozygote. Arrows represent means of distribution.

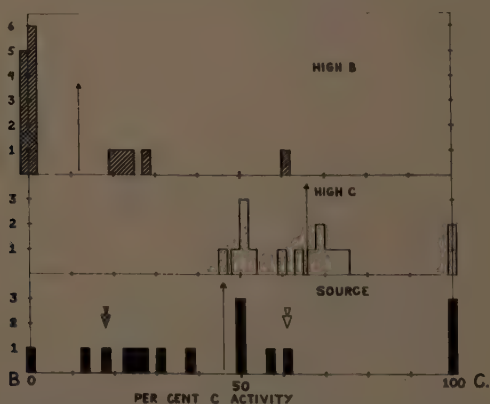


FIGURE 17. Distributions of lineages derived from different E-1B + C subclones. Arrows pointing up represent means of distributions; sources of two top distributions indicated by arrows pointing down.

butions of subclones that resulted. The mean activities were similar in all three arrays of subclones.

When E-1B + C subclones with different percentages of C activity were deliberately selected and expanded, the resulting distributions of cell lineages were markedly dissimilar (FIGURE 17). Two subclones were selected from the array shown on the bottom graph. The middle graph records the distribution of cell lineages obtained from a subclone with 62 per cent C activity, the top graph that obtained from a subclone with 18 per cent C activity. Only one stable type of cell lineage (either E-1B or E-1C) was produced by these subclones with eccentric activities.

Adequate kinetic data are not available for the *E-1* esterases; however, similar patterns of vegetative assortment were observed at the *mt* locus (Allen and Nanney, 1958) and at the *H* serotype locus (Nanney and Dubert, 1960). These patterns of vegetative assortment were interpreted in terms of a particulate segregation model, developed by Schensted (1958). The macronucleus was postulated to contain diploid subnuclei, which could differentiate to yield a heterogeneous macronucleus. Stable cell lines would arise as a consequence of

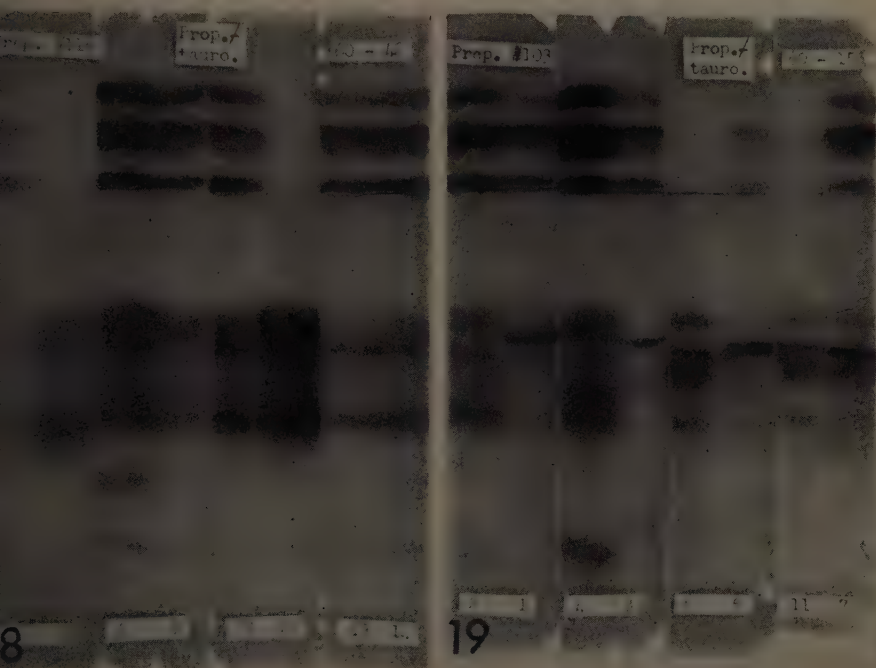


FIGURE 18. *E-1* esterases of 8 F2 pairs from a cross of F1 (*E-1B* × *E-1B*). Substrate: *p*-naphthyl propionate with sodium taurocholate. Incubated 4 hours.

FIGURE 19. *E-1* esterases of 8 F2 pairs from a cross of F1 (*E-1C* × *E-1C*). Substrate: *p*-naphthyl propionate with sodium taurocholate. Incubated 4 hours.

random assortment of the differentiated subnuclei to daughter cells during division. The kinetics of segregation were identical for the *mt* and *H* loci.

The differentiated cell lines at the *mt* and *H* loci were stable only during vegetative reproduction. The differentiated cell lines at the *E-1* locus were also stable during vegetative reproduction. Tests of the persistence of these differentiations were made by crossing all three phenotypes (*E-1B* + *C*, *E-1B*, *E-1C*) in all six phenotypic combinations.

Crosses of the most extreme combinations (*E-1B* × *E-1B* and *E-1C* × *E-1C*) gave rise to pairs of all three classes of phenotypes (FIGURES 18 and 19). This result was obtained regardless of phenotypic combination (TABLE 5). Thus, the differentiations did not persist after conjugation—and all three classes of pairs appeared in the progeny as expected of a cross of two heterozygotes. In

order to test possible deviations from a 2:1:1 ratio, crosses most similar in character were lumped, since some of the crosses were small. Although qualitative trends are found among different sets of crosses, no set showed a significant departure from a 2:1:1 ratio, nor did the four sets show significant heterogeneity. Hence, the differentiations not only disappear following conjugation but they exert no significant cytoplasmic influence upon the system.

One final observation on the heterozygote should be mentioned, although the data are only *suggestive*. That is, regardless of phenotype, the total of the activities of the two groups approached a similar value. The totals for individual subclones varied (range 180 to 455 U.), but this range undoubtedly reflected differences in protein concentration between preparations. The mean value for 12 E-1B subclones was 300 U., for 8 E-1C subclones was 240 U., and for 44 E-1B + C subclones was 333 U. The lower value for the E-1C subclone

TABLE 5
CROSSES OF HYBRID CLONES WITH DIFFERENT E-1 PHENOTYPES

Parental phenotypes	Esterases of offspring:			Total No. pairs	Fit to 2:1:1
	B + C	B	C		
(B + C) × (B + C)	31	13	16	60	p = 0.9
B × (B + C)	14	7	2	23	p = 0.5
B × B	7	5	4	16	
	21	12	6	39	
C × (B + C)	2	2	2	6	p = 0.5
C × C	20	5	10	35	
	22	7	12	41	
B × C	14	13	10	37	p = 0.3
Total	88	45	44	177	p = 0.99

may not be real because of the way in which Densitometer recordings were measured. A maximum value of light absorption was recorded for each isozyme. Such a procedure would not take into account differences in the width of individual zones in the starch. The E-1C isozymes typically are wider and less distinct than the E-1B isozymes. Thus these maximum values tend to underestimate the E-1C activities. The values of heterozygous clones were similar to those of homozygous clones, with the E-1C homozygote having a slightly lower value.

Additivity of these isozymes toward a similar value, irrespective of genotype and phenotype, suggests that only a certain amount of a protein is made. This suggestion leads to certain speculations concerning the nature of an isozyme, to be discussed below.

Discussion

The nature of an isozyme. The distinction between a family of enzymes and an isozymic series largely rests upon the degree of specificity which is observed.

Markert and Møller (1959) defined a family of enzymes as one comprising a group of different enzymes with broad and overlapping substrate specificities. The members of an isozymic series, on the other hand, have a more restricted spectrum of specificity. Observations on the esterases of variety 1 of *T. pyriformis* confirm this distinction. Considerable heterogeneity in enzymatic activities against four substrates and an inhibitor and an activator are found between different members of the family, although many of these members show overlapping specificities. Some of these members are under the control of two unlinked loci. Two of these members are made up of an isozymic series, which show identical specificities under the conditions employed, and the two isozymic sets are under the control of alleles at a single locus.

Only a few genetic studies have been made on the esterases in other organisms. A polygenic mode of inheritance was postulated for the cerebral cholinesterases of the rat (Roderick, 1960). Single gene differences were postulated for piglet arylesterase (Augustinsson and Olsson, 1959), human pseudocholinesterase (Harris *et al.*, 1960) and maize aliesterase (Schwarz, 1960). No genetic studies have as yet been reported on an isozymic series of esterases, but an exciting study is in progress on an isozymic series of alkaline phosphatases in *Escherichia coli* (Bach *et al.*, 1961).

In *E. coli* starch gel electrophoresis resolved the phosphatases into five electrophoretically distinct components, although a *single protein* is implicated by Tiselius electrophoresis, sedimentation, and immunological characterization. Chromatographic separation on DEAE cellulose columns showed a single peak, but some heterogeneity of the different isozymes within this peak. Single mutations in the phosphatase gene—or growth under conditions of enzyme repression—eliminated all of the isozymes. Reverse mutations included a number of mutants with altered electrophoretic mobilities, although the number and spacing of the isozymes was retained in the mutants. All the mutants that have been tested genetically show changes within the phosphatase gene. The genetic observations are thus similar to those obtained with the E-1 esterase isozymes in *Tetrahymena*, although on a much more definitive scale. Quantitative variations in these isozymes were observed with growth on different media similar to the sorts of variations seen in *Tetrahymena*.

The finding of single gene control of an isozymic set limits speculation concerning the nature of an isozyme, at least in the context of the genetic dogma as it now stands. According to the dogma, a certain prescribed sequence of events transpires from gene to protein: the DNA code is imprinted in RNA, which translates the message into particular amino acid sequences in the protein. These sequences then are supposed to determine a secondary and tertiary structure of the protein. A problem arises in interpreting the electrophoretic differences of a protein with the same enzymatic activity under such a rigid dogma. Markert and Møller (1959) suggested that either "the protein forming mechanism itself is subject to slight structural variations with a consequent variation in its products" or that the protein could be altered by its site of attachment within the cell. The latter interpretation is more consistent with genetic dogma and also certain experimental observations.

Preliminary studies on cell fractions in *Tetrahymena* suggest that each isozyme may have a different site of attachment within the cell. Observations in

two other organisms support this idea. Nace *et al.* found that all four lactic acid dehydrogenase (LDH) isozymes are present in the coelomic eggs of frogs and that two of these isozymes, examined in detail, are localized in different regions of the egg. J. M. Allen, 1961, working with the LDHs of the mouse, was able to localize one of these to the apex of epithelial cells of the body of the epididymis.

A genetic interpretation of this idea was suggested by Levinthal (Spiegelman, 1960). This rests on the assumption that an isozyme is a molecule composed of several proteins. The active site of this molecule could be composed of one polypeptide, the tail of another polypeptide. Since isozymes have similar enzymatic properties, they must contain the same polypeptide in the active site. The tail polypeptides, which would govern the specificity of attachment, could differ, and these could be governed by several different genes.

Such an interpretation would lead to polygenic inheritance for a series of isozymes. Some isozymic series may well turn out to show this type of inheritance, but in at least two cases (*Tetrahymena* and *E. coli*) this interpretation works only if the following is assumed: that the strains are isogenic for a gene controlling the polypeptide of the active site and also for genes controlling the tail polypeptides, but that they differ in genes controlling a third polypeptide of unknown biological properties.

Since so many assumptions are needed in order to fulfill this interpretation, two other avenues of approach will be pursued. The first approach adheres to genetic dogma and achieves a way out of the dilemma through polymerization. If a single polypeptide formed polymers of varying size, different specificities of attachment might arise as a consequence of the different tertiary structures of the polymers. The active site, however, must not be affected either by gene substitution or by polymerization. There is evidence for the formation of polymers in systems of this kind in two studies. Smithies (Ingram, 1960) reported that the several electrophoretic components of the haptoglobins in man were stable polymers, which could be resolved into single components by treatment with mercaptoethanol, iodoacetamide, and urea. In maize certain aliesterases are under the control of three alleles at a single locus, and each heterozygote forms a hybrid molecule in addition to the parental molecules (Schwarz, 1960). The amounts of enzymatic activity were additive in the different genotypes, but distributed among each set of components in a manner suggesting that each molecule was a dimer.

The results of Bach *et al.*, 1961, clearly indicate that different states of aggregation of the phosphatase protein do *not* occur. A single species of protein is involved and it does not form polymers, yet this protein shows electrophoretic heterogeneity in starch gels. A way out of this dilemma may be found by permitting *slight* structural variations in the tertiary structure of a protein. Thus the amino acid sequences of a polypeptide would usually result in a certain protein configuration, but in response to different microecological factors, slight deviations in form might be produced. The number of configurations that would be stable would probably be very limited: perhaps by the number of sites of attachment in the cell. These configurations might provide the necessary specificity of attachment to particular sites so that the enzyme would be

channeled (Vogel, 1955) to particular reaction sequences taking place in different parts of the cell.

Although no data are yet available as to the physicochemical nature of the isozymes in *Tetrahymena*, the possible additivity of the isozymes in different genotypes suggests that only a certain amount of protein is made, which is then "distributed" among the different isozymes of a group proportionate to the degree of allelic activity in the cell lineage. "Distribution" could mean polymerization or it could mean alteration in configuration of the original molecule.

The different configurations may be involved in the attachment of the enzyme to specific sites within the cell. Differences in activity of an isozyme under various conditions of growth, observed in *Tetrahymena* and *E. coli*, could reflect differences in the binding of this isozyme to its site of attachment. Facilitation or interference with this binding would lead to the observation of more or less enzyme becoming attached to a particular site. A corollary of this hypothesis is that certain isozymes in each group might show similar behavior under a given condition of growth. The changes during the growth cycle in *Tetrahymena* are not so easily explained, but those observed under two other conditions of growth do show correlations. Thus, the cathodal isozymes of each group were more active with growth at 16° C., while the anodal isozymes of each group were more active with starvation and/or mating.

Nuclear differentiation in Tetrahymena. Previous studies of variety 1 of *T. pyriformis* have implicated the macronucleus and, more specifically, the sub-nucleus as the locus of nuclear differentiation in this organism (Allen and Nanney, 1958; Nanney and Allen, 1959; Nanney and Dubert, 1960). Analysis of the mating type locus and the *H* serotype locus showed some features that were different but many features that were remarkably similar.

At each of these loci are a series of alleles. At the *H* locus each allele determines a ciliary antigen of slightly different specificity at 20–30° C. (Nanney and Dubert, 1960). At the mating type locus each allele permits the expression of several of the seven mating types, each allele differing in which of the seven types are expressed and the frequencies in which they appear at a particular temperature (Nanney, 1959, 1960a; Nanney *et al.*, 1955). The heterozygote at both these loci has the potentiality of expressing all parental phenotypes.

The phenotypes at both these loci appear to be determined at the level of the caryonide, but this determination is more obvious in the case of the mating types. The correlation of the distributions of the mating types and the new macronuclei within a pair was observed almost 25 years ago by Sonneborn in a related ciliate, *Paramecium aurelia* (Sonneborn, 1960). Any experimental condition that interfered with the formation or distribution of new macronuclei resulted in alteration of the appearance or frequency of a mating type. Thus, it was concluded that the new macronuclei become differentiated during their formation, so that they control different phenotypes.

A more precise localization of the control of the mating types was demonstrated in *Tetrahymena*. Certain caryonides are unstable and mixed in phenotype (Nanney and Caughey, 1955; Allen and Nanney, 1958). They give rise to cell lines that are also unstable and to two or more cell lines that are stable for

different mating types. An analysis of unstable caryonides giving rise to two stable lines showed that the kinetics of formation of the stable lines were the same, regardless of the two types that were present. At equilibrium the total rate of fixation was found to be 0.0113 per fission and the two types were produced at equal rates. These results were consistent with a particulate segregation model that assumed that the macronucleus consisted of diploid subnuclei, which could differentiate and give rise to a heterogeneous macronucleus. Random segregation of these differentiated subnuclei to daughter macronuclei would occasionally lead to the production of a macronucleus pure for one type of subnucleus. Such cell lines would be stable. The rate per fission (R_f) at which stable lines are produced was shown by Schensted (1958) to be related to the total number (N) of subnuclei in the macronucleus; thus $R_f = 1/(2N - 1)$. Where $R_f = 0.0113$, the number of subnuclei in a newly divided cell is 45.

Similar observations were made on heterozygotes at the *H* locus (Nanney and Dubert, 1960). Heterozygous caryonides are unstable and give rise to cell lineages, some of which are unstable and express both alleles at the *H* locus, and others which are stable and express only one of the two alleles. The rate of formation of these stable cell lines was identical to that determined for the mating type system.

An analysis of the *H* heterozygote suggests that the site of nuclear differentiation in *Tetrahymena* is not only localized within the subnucleus but to the chromosomes themselves. One allele in each subnucleus of a heterozygote is suppressed, but which allele is expressed varies in different subnuclei. Cells with heterogeneous macronuclei express both alleles and are unstable. Cells with macronuclei containing subnuclei all suppressed for the same allele, express the other allele and are stable.

Differentiation occurs only in heterozygotes at the *H* locus. At the *mt* locus differentiation also occurs in homozygotes, and as many as four phenotypes may occur in different cell lineages of a single caryonide. As Nanney and Dubert (1960) point out, this observation makes gene segregation within the macronucleus very improbable and supports the hypothesis that the differentiations occur in different diploid subnuclei.

The differentiations at both the *mt* and *H* loci are permanent during the remainder of the vegetative existence of the cell. Those at the *H* locus even persist after a period of exposure to conditions that result in the nonfunctioning of the *H* locus. However, the differentiations at both the *mt* and *H* loci do not persist after conjugation.

Many features of the *mt* and *H* loci are shared by the *E-1* locus. Like the *H* locus, the two alleles at the *E-1* locus, when present in a heterozygote, vary in expression within a caryonide. Unstable cell lineages, which express both alleles, give rise to stable cell lines expressing only one of the two alleles. Adequate kinetic data are lacking for the *E-1* locus, but the observations on heterozygous clones showing that they varied qualitatively and quantitatively in their capacity to produce stable cell lines are consistent with crude observations made on the mating type and serotype systems. Like the differentiations at the *mt* and *H* loci, those at the *E-1* locus persist only during vegetative reproduction.

An analysis of vegetative assortment at the *mt* and *H* loci showed that each

system is independent of the other (Nanney, 1960*b*). They are unlinked genetically as well as phenotypically. These two systems (and probably the E-1 esterases, too) converge only in that their site of differentiation—the subnucleus—is the same. Hence, their common kinetic properties arise from a common physical organization of the subnuclei within the macronucleus. The secret of the control of these individual phenotypes lies within these subnuclei: probably on the chromosomes themselves.

Cellular differentiation and isozymes. Cellular differentiation results in striking differences in patterns of activity of the LDH isozymes (Markert and Møller, 1959; Tsao, 1960). Different patterns were observed between similar adult tissues and tissue cultures of different species, between different adult tissues of the same species, and between similar adult and embryonic tissues of the same species. Changes in pattern of esterase activity were also observed during ontogeny in mouse tissues (Markert and Hunter, 1959). Contrary to these findings, only quantitative differences in the activity of the esterase isozymes were observed between cells of the same genotype in *Tetrahymena* (Allen, 1960*a*). Clonal analyses revealed that all the isozymes of a group (or groups) were present in the cells of the three genotypes.

In higher organisms some of the isozymic changes during ontogeny could be due to differential gene activity where a polygenic mode of inheritance is implicated. In these cases it is simplest to assume two states for a gene: either it is "on" or it is "off". A given pattern of cellular differentiation would then result from a certain combination of genes that are "on". Where single gene control is involved, the isozymic changes could reflect interference at several different points in the sequence from gene to protein.

In *Tetrahymena* several different phenotypes are often associated with a single genotype and, in the cases examined, the control of these differences resides within the subnucleus and is probably chromosomal. It is possible that interference with DNA occurs, perhaps in its association with RNA or nuclear proteins, to give rise to several different states of a gene besides its "on" or "off" states. Nanney (1960*a*) speculated that different folding configurations of material associated with the chromosomes could give rise to several molecular species, each of which could ultimately give rise to a different phenotype. These configurations might replicate—along with the chromosome—during the vegetative existence of the cell, but they would be eliminated at the onset of meiosis. Brink (1960) suggested that one of the prime functions of meiosis might be to strip the chromosomes of associated materials acquired during the vegetative life of the cell.

A similar type of control may be exerted in some of the isozymic series, if nuclear differences can be demonstrated. However, for systems that do not show nuclear differences—as in the case of the esterase isozymes in *Tetrahymena*—the level of control appears to be further removed from the gene. In such systems some type of control may be exerted on the channeling of particular isozymes to their sites of attachment within the cell. Microecological differences arising from different cellular associations and environments could determine the stability of particular configurations and the configurations at the attachment sites. Under certain conditions these configurations could persist with a high degree of stability.

Summary

The esterases of variety 1 of *T. pyriformis* can be resolved into a number of zones by starch gel electrophoresis. These zones can be separated into two classes on the basis of substrate specificity and reaction to eserine sulfate and sodium taurocholate. At pH 7.5 to 9.5 one of these classes includes two groups of isozymes, which have similar substrate specificities and are inhibited by eserine and activated by taurocholate. The B group of isozymes is found in inbred strains A, B, and D, while the C group is limited to strain C. Each group of isozymes functions as a unit during vegetative and sexual reproduction. Isozymic activities vary under different growth conditions and, as preliminary studies indicate, in different parts of the cell.

Crosses between B and C cells produce hybrids with both groups of isozymes (B + C). However, hybrids vary within the clone in their expression of the two groups, and subclones phenotypically B or C can be selected. Hybrids, in various phenotypic combinations, when intercrossed, give rise to three classes of F₂ pairs, B + C, B and C in a 2:1:1 ratio. Backcrossed to parental cells, only two classes appear, B + C and B (or C) in a 1:1 ratio. Thus the two groups of isozymes are controlled by alleles at a single locus (*E-1*). A homozygote expresses only the B or only the C group. A heterozygote has the potentiality of expressing both groups, but it becomes differentiated (probably by allelic suppression) so that it expresses either the B or the C group. These differentiations persist only during the vegetative life of the cell.

Evidence will also be presented for a second locus (*E-2*) governing two members of the other class of esterases. *E-1* and *E-2* appear to be unlinked loci.

Since a single gene was implicated in the control of a set of isozymes, a single protein appears to be involved. This inference leads to certain speculations concerning the structure and function of an isozyme. An isozyme could arise either by polymerization or by structural alteration of a single protein. These configurations could endow the isozymes with differences in specificity necessary for their attachment to certain sites in the cell as a prerequisite for their function in particular reaction sequences.

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References

- ALLEN, J. M. 1961. Multiple forms of lactic dehydrogenase in tissues of the mouse, their specificity, cellular localization and response to altered physiological conditions. *Ann. N.Y. Acad. Sci.* **94**: 937-951.
- ALLEN, S. L. 1958. Cytochemical localization of enzymes in sexual strains of the Protozoan, *Tetrahymena pyriformis*. *Anat. Record.* **131**: 526-527.
- ALLEN, S. L. 1960a. Inherited variations in the esterases of *Tetrahymena*. *Genetics.* **45**: 1051-1070.
- ALLEN, S. L. 1960b. Abnormal segregation at the mating type locus in variety 1 of *Tetrahymena pyriformis*. *J. Protozool.* **7**(Suppl.): 15.
- ALLEN, S. L. & D. L. NANNY. 1958. An analysis of nuclear differentiation in the selfers of *Tetrahymena*. *Am. Naturalist.* **92**: 139-160.
- AUGUSTINSON, K.-B. & B. OLSSON. 1959. Esterases in the milk and blood plasma of swine. 2. Activities at different stages during the lactation and suckling periods, and plasma arylesterase as a gene-controlled enzyme. *Biochem. J.* **71**: 484-492.

- BACH, M. L., E. R. SIGNER, C. LEVINTHAL & I. W. SIZER. 1961. The electrophoretic patterns of alkaline phosphatase from various *E. coli* mutants. *Federation Proc.* **20**: 225.
- BRINK, R. A. 1960. Paramutation and chromosome organization. *Quart. Rev. Biol.* **35**: 120-137.
- HARRIS, H., M. WHITTAKER, H. LEHMANN & E. SILK. 1960. The pseudocholinesterase variants. Esterase levels and dibucaine numbers in families selected through suxamethonium sensitive individuals. *Acta Genet. et Statist. Med.* **10**: 1-16.
- HUNTER, R. L. & C. L. MARKERT. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science*. **125**: 1294-1295.
- INGRAM, V. M. 1960. The genetic control of protein structure. In *Genetics: Genetic Information and the Control of Protein Structure and Function*. : 65-176. H. E. Sutton, Ed. Josiah Macy, Jr. Foundation. New York, N.Y.
- LEVINTHAL, C. 1960. Discussion in S. Spiegelman. Factors modulating the biochemical expression of genetic systems. : 189-190. In *Genetics: Genetic Information and the Control of Protein Structure and Function*. H. E. Sutton, Ed. Josiah Macy, Jr. Foundation. New York, N.Y.
- MARKERT, C. L. & R. L. HUNTER. 1959. The distribution of esterases in mouse tissues. *J. Histochem. Cytochem.* **7**: 42-49.
- MARKERT, C. L. & F. MÖLLER. 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci. U.S.* **45**: 753-763.
- NACE, G. W., T. SUYAMA & N. SMITH. Early development of special proteins. Symposium of Internal Inst. of Embryol. Pallanza, Italy. In press.
- NANNEY, D. L. 1958. Epigenetic control systems. *Proc. Natl. Acad. Sci. U.S.* **44**: 712-717.
- NANNEY, D. L. 1959. Genetic factors affecting mating type frequencies in variety 1 of *Tetrahymena pyriformis*. *Genetics*. **44**: 1173-1184.
- NANNEY, D. L. 1960a. Temperature effects on nuclear differentiation in variety 1 of *Tetrahymena pyriformis*. *Physiol. Zööl.* **33**: 146-151.
- NANNEY, D. L. 1960b. The relationship between the mating type and the H serotype systems in *Tetrahymena*. *Genetics*. **45**: 1351-1358.
- NANNEY, D. L. & S. L. ALLEN. 1959. Intranuclear co-ordination in *Tetrahymena*. *Physiol. Zööl.* **32**: 221-229.
- NANNEY, D. L. & P. A. CAUGHEY. 1955. An unstable nuclear condition in *Tetrahymena pyriformis*. *Genetics*. **40**: 388-398.
- NANNEY, D. L., P. A. CAUGHEY & A. TEFANKJIAN. 1955. The genetic control of mating type potentialities in *Tetrahymena pyriformis*. *Genetics*. **40**: 668-680.
- NANNEY, D. L. & J. M. DUBERT. 1960. The genetics of the H serotype system in variety 1 of *Tetrahymena pyriformis*. *Genetics*. **45**: 1335-1349.
- PREER, J. R., JR. & L. B. PREER. 1959. Gel diffusion studies on the antigens of isolated cellular components of *Paramecium*. *J. Protozool.* **6**: 88-100.
- RODERICK, T. H. 1960. Selection for cholinesterase activity in the cerebral cortex of the rat. *Genetics*. **45**: 1123-1140.
- SCHENSTED, I. V. 1958. Appendix: model of subnuclear segregation in the macronucleus of ciliates. *Am. Naturalist*. **92**: 161-170.
- SCHWARZ, D. 1960. Genetic studies on mutant enzymes in maize: synthesis of hybrid enzymes by heterozygotes. *Proc. Natl. Acad. Sci. U.S.* **46**: 1210-1215.
- SEAMAN, G. R. 1951. Localization of acetylcholinesterase activity in the protozoan, *Tetrahymena geleii* S. *Proc. Soc. Exptl. Biol. Med.* **76**: 169-170.
- SMITHIES, O. 1960. Discussion in V. M. Ingram. The genetic control of protein structure. In *Genetics: Genetic Information and the Control of Protein Structure and Function*. : 129-136. H. E. Sutton, Ed. Josiah Macy, Jr. Foundation. New York, N.Y.
- SONNEBORN, T. M. 1957. Breeding systems, reproductive methods and species problems in protozoa. In *The Species Problem*. : 155-324. E. Mayr, Ed. A.A.A.S. Symposium. Washington, D.C.
- SONNEBORN, T. M. 1960. The gene and cell differentiation. *Proc. Natl. Acad. Sci. U.S.* **46**: 149-165.
- SPIEGELMAN, S. 1960. Factors modulating the biochemical expression of genetic systems. In *Genetics: Genetic Information and the Control of Protein Structure and Function*. : 177-218. H. E. Sutton, Ed. Josiah Macy, Jr. Foundation. New York, N.Y.
- TS'AO, M. U. 1960. Heterogeneity of tissue dehydrogenases. *Arch. Biochem. Biophys.* **90**: 234-238.
- VOGEL, H. J. 1955. On the glutamate-proline-ornithine interrelation in various microorganisms. In *A Symposium on Amino Acid Metabolism*. : 335-346. W. D. McElroy & H. B. Glass, Eds. The Johns Hopkins Press. Baltimore, Md.
- WATSON, J. D. 1960. Current concepts of the fine structure of chromosomes and the nature of the coding mechanism. In *Genetics: Genetic Information and the Control of Protein Structure and Function*. : 11-64. H. E. Sutton, Ed. Josiah Macy, Jr. Foundation. New York, N.Y.

VARIOUS FORMS OF D- AND L-LACTATE DEHYDROGENASES IN YEAST

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The catalytic oxidation of racemic lactate by yeast extracts, which is independent of added coenzymes, has been known for more than 30 years (Bernheim, 1928). It was not realized until recently, however, that several lactate dehydrogenases may be present in yeast extract. In addition to L-lactate cytochrome *c* reductase, which has been known for many years (Nygaard, 1961), three D-specific lactate dehydrogenases are present in yeast: they are (1) D-lactate dehydrogenase of yeast grown anaerobically (Lindenmeyer and Smith, 1957; Labeyrie *et al.*, 1959); (2) D-lactate cytochrome *c* reductase (Nygaard, 1958); and (3) D-lactate dehydrogenase of yeast grown aerobically (Nygaard, 1961a). Lactate is the most effective substrate for all of these enzymes, either giving the highest maximal rate, or having the lowest apparent Michaelis constant; several other alpha-hydroxycarboxylic acids are oxidized however; the stereospecificity is absolute. The acceptor specificities are widely different for the different enzymes; for example, D-lactate dehydrogenase of anaerobic yeast does not reduce cytochrome *c*, whereas D-lactate cytochrome *c* reductase has a high specificity for cytochrome *c* as acceptor.

The physiological function of the various yeast-lactate dehydrogenases is unknown; attempts have been made to show that they are interrelated biosynthetically, but no direct evidence has been presented (Kattermann and Slonimski, 1960; Nygaard, 1961b). At the present time the composition and the properties of the yeast-lactate dehydrogenases are studied in a comparative manner. The present report concerns (1) the binding of the prosthetic groups, (2) the reversible inactivation of D- and L-lactate cytochrome *c* reductase, and (3) the effect of some inhibitors at the substrate and acceptor sites.

The enzymic preparations, materials, and methods used in this study have been described elsewhere (Nygaard, 1959, 1960, 1961).

The Binding of the Prosthetic Groups

L-Lactate cytochrome *c* reductase. This enzyme is also termed cytochrome *b*₂; it contains equimolar quantities of flavin mononucleotide (FMN) and protoheme (Appleby and Morton, 1954). The fluorescence of FMN is quenched in the active enzyme, but it appears on storage, and this is accompanied by irreversible inactivation. FMN dissociates rapidly from the protein by the addition of (1) thiol reagents (Armstrong *et al.*, 1960), and (2) acetic anhydride, applied in small quantities at neutral pH. These observations cannot be taken as strong evidence, however, for the participation of sulfhydryl (SH) groups and amino-acid groups in the binding of FMN; the dissociation could be caused by denaturation of the holoenzyme, for example, by unfolding of the peptide chain.

D-Lactate cytochrome *c* reductase. The fluorescence of the prosthetic group flavin adenine dinucleotide (FAD), is quenched in the native enzyme, but it

appears during storage, and this is accompanied by denaturation of the enzyme (Nygaard, 1961a). FAD dissociates rapidly from the protein by the addition of (1) silver nitrate, and (2) acetic anhydride, applied at neutral pH. However, it is possible to inactivate the enzyme with silver nitrate before the fluorescence appears; this is done by the addition of small quantities of the reagent. D-Lactate cytochrome *c* reductase may, therefore, contain essential thiol groups that are not involved in the binding of FAD.

Dialysis of D-lactate cytochrome *c* reductase against 0.01 *M* EDTA or 0.001 *M* *o*-phenanthroline at pH 6.8 increases the rate of inactivation of the enzyme; no reactivation has been achieved by the addition of various metals.

D-Lactate dehydrogenase of anaerobic yeast. This enzyme is a metalloflavoprotein (Boeri *et al.*, 1960; Curdel *et al.*, 1959). FAD protects against inactivation of the enzyme by atabrine, and certain preparations obtained during the purification procedure are stimulated by FAD; FMN has none of these effects (Baudras *et al.*, 1960). These observations indicate that the prosthetic group is FAD, which is loosely bound to the protein. The enzyme is inactivated by dialysis against chelating agents and reactivated by several divalent metals.

D-Lactate dehydrogenase of aerobic yeast. This is an extremely labile enzyme, with FMN as the prosthetic group (Nygaard, 1961a). The dissociation of FMN from the protein, as measured by the appearance of fluorescence, roughly parallels the decrease of activity. D-Lactate decreases the rate of dissociation; it is increased by dilution of the enzyme.

In conclusion, none of the lactate dehydrogenases of yeast have been split reversibly into flavin nucleotide and apoenzyme under controlled conditions. The nucleotides appear to be loosely bound to labile proteins, and the specific linkages involved in the binding are unknown.

Reversible Inactivation of D- and L-Lactate Cytochrome c Reductase

D- and L-lactate cytochrome *c* reductase are stabilized by their respective substrates: D- and L-lactate. The acceptor, cytochrome *c*, on the other hand, appears to form an inactive complex with ferricytochrome *c*, which dissociates by dilution of the enzyme-cytochrome *c* mixture. When dialyzed preparations of the enzymes are incubated with 1×10^{-5} *M* ferricytochrome *c* at 5° C. for some days, the activity with cytochrome *c* as acceptor, as measured by initial reaction rates, is decreased, while the activity with ferricyanide (of the L enzyme) remains constant; reactivation takes place during the assay procedure, or it can be accomplished by dilution of the enzyme with water or buffer, in the absence or presence of substrate. High concentrations of cytochrome *c* (1×10^{-3} *M*) prevent the reactivation; crystalline serum albumin (100 mg. per ml.) does not have this effect. The reactivation phenomenon is not observed if ferricyanide is used as acceptor for L-lactate cytochrome *c* reductase. The relative activities with cytochrome *c* and ferricyanide are 1:1 for the freshly prepared enzyme, as well as for the reactivated preparations. Reactivation experiments with D- and L-lactate cytochrome *c* reductase are shown in FIGURES 1 and 2 respectively.

Since the reversible inactivation appears to be caused by the formation and dissociation of an inactive enzyme-ferricytochrome *c* complex, one may explain

the results by considering a possible reaction mechanism of these two enzymes, which is as follows (Nygaard, 1961c; Morton *et al.*, 1961):

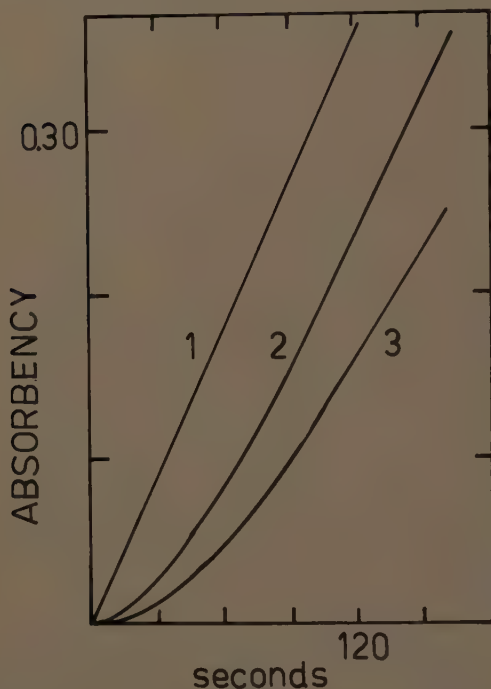


FIGURE 1. The enzymic activity of D-lactate cytochrome *c* reductase, which has been incubated with $1 \times 10^{-6} M$ ferricytochrome *c* for 5 days at 5° . The composition of the assay medium is as follows: ferricytochrome *c*, $3 \times 10^{-5} M$, D-lactate, $5 \times 10^{-3} M$; in a total volume of 2 ml. Na-phosphate, 1/2 0.01 plus 0.001 *M* EDTA, pH 6.8; 23° . The reaction rates are obtained from the absorbency change at $550 m\mu$ with time:

(1) Twenty-five $\mu l.$ of the enzymes was diluted with 100 $\mu l.$ of the buffer 6 min. before added to the assay medium.

(2) The enzyme was added directly to the assay medium, without previous dilution. An identical curve was obtained when 25 $\mu l.$ of the enzyme was diluted with 50 $\mu l.$ of the buffer plus 50 $\mu l.$ of $1.2 \times 10^{-3} M$ ferricytochrome *c* 6 min. before added to the assay medium (buffer + D-lactate).

(3) As in the first step, but the assay medium contained 0.02 *M* NaCl.

(*E* and *EH* denote the oxidized and reduced form of the enzyme respectively; *SH*, lactate; *S*, pyruvate; *CyFe⁺⁺⁺* and *CyFe⁺⁺*, the oxidized and reduced form of cytochrome *c* respectively). The postulated, inactive complex *E.CyFe⁺⁺⁺* may have to be dissociated before the oxidized form of the enzyme, *E*, can react with lactate, and the dissociation, which takes place by dilution, could be prevented by *CyFe⁺⁺⁺*, according to the law of mass action. Since ferricyanide and cytochrome *c* may react with different acceptor sites of L-lactate cytochrome *c* reductase (Nygaard, 1961*d*), the ferricyanide site may not be occupied

in the $E.CyFe^{+++}$ complex; hence the complex could be active, without dissociation, in the L-lactate ferricyanide system, which may follow a reaction mechanism different from that proposed with cytochrome c (Morton *et al.*, 1961).

Interaction at the Substrate Site

D- and L-lactate cytochrome c reductase are inhibited competitively by fatty acids, and the inhibition increases with the carbon chain of the inhibitor; log

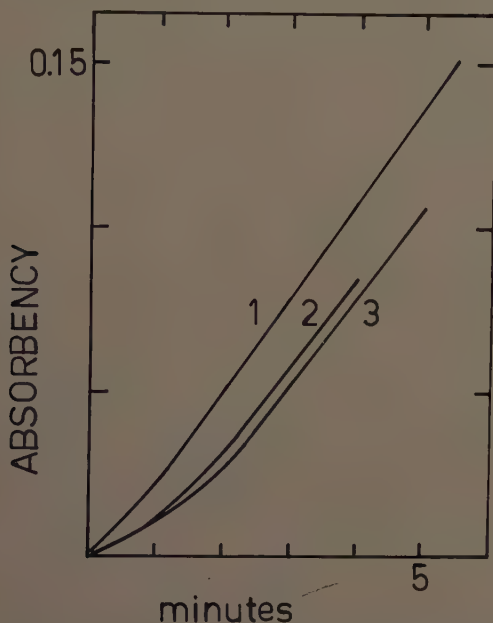


FIGURE 2. The enzymatic activity of L-lactate cytochrome c reductase, which has been incubated with $1 \times 10^{-5} M$ ferricytochrome c for 5 days at $5^{\circ} C$. All the conditions are as described in FIGURE 1:

- (1) Twenty-five μl . of the enzyme was diluted with 100 μl . of buffer 6 min. before added to the assay medium.
- (2) Twenty-five μl . of the enzyme was diluted with 50 μl . of the buffer plus 50 μl . of $1.2 \times 10^{-3} M$ ferricytochrome c 6 min. before added to the assay medium.
- (3) The enzyme was added directly to the assay medium, without previous dilution.

K_I (K_I , the dissociation constant of the enzyme-inhibitor complex) has been found to be a linear function of the number of $-CH_2-$ groups of the various fatty acids (Nygaard, 1961*d*). From these observations the free energy of interaction, $\Delta F = RT \ln K_I$, of (1) the $-CH_2-$ group with the enzyme surface, and (2) the carboxylic ion with a postulated charge at the substrate site, have been calculated. The former ΔF_{-CH_2-} , is -370 cal. per mole for both enzymes; the latter ΔF_{COO-} , is -2800 cal. per mole for D-lactate cytochrome reductase, and -880 cal. per mole for L-lactate cytochrome c reductase.

The fact that L-lactate cytochrome c reductase reduces both cytochrome c and ferricyanide has been used to study the effect of the acceptor on the free energy of interaction of fatty acids at the substrate site. The inhibition is

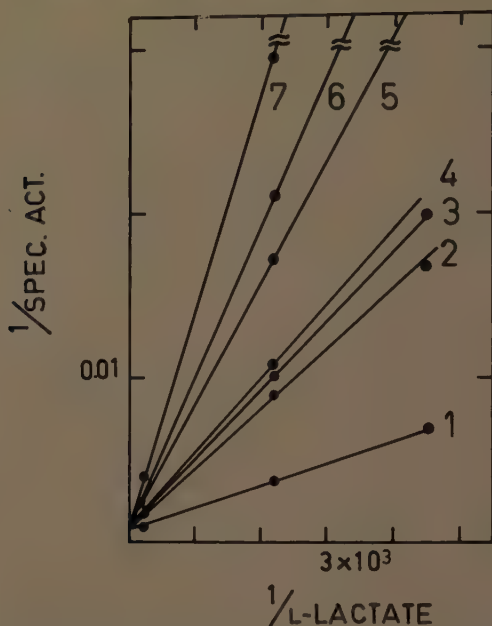


FIGURE 3. Substrate-competitive inhibition of L-lactate cytochrome *c* reductase by butyrate, caprylate, and laurate. Acceptor, $9 \times 10^{-4} M$ ferricyanide; $5 \times 10^{-3} M$ L-lactate; buffer, phosphate, $\Gamma/2$ 0.1, pH 6.8 plus 0.001 *M* EDTA; 23° C.: (1) no inhibitor added; (2) 0.05 *M* butyrate; (3) 0.0075 *M* caprylate; (4) 0.0005 *M* laurate; (5) 0.15 *M* butyrate; (6) 0.01 *M* laurate; and (7) 0.3 *M* butyrate. From the degree of inhibition by butyrate and laurate at various concentrations of the inhibitors, the inhibition can be shown to be of the EI type; one mole of inhibitor combines with each substrate site.

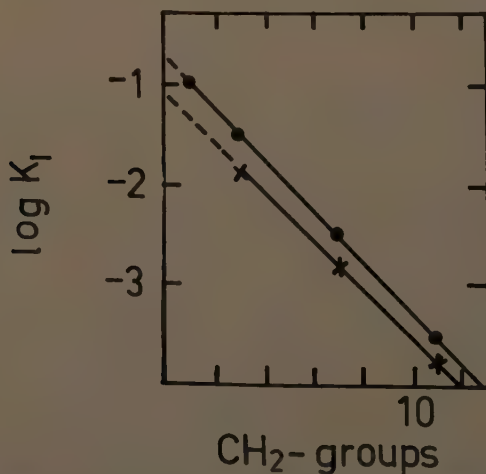


FIGURE 4. The logarithm of the inhibitor constant, K_I , as a function of the number of $-CH_2-$ groups of the fatty acids. Key: X—X—X, the L-lactate ferricyanide reductase system (the experimental values are taken from FIGURE 3); ●—●—●, the L-lactate cytochrome *c* reductase system. Reproduced by permission of the *Journal of Biological Chemistry* (Nygaard, 1961d).

competitive also in the ferricyanide system (FIGURE 3), but K_I is lower when ferricyanide is introduced as acceptor; parallel lines are obtained for the two acceptor systems when $\log K_I$ is plotted against the number of $-\text{CH}_2-$ groups of the inhibitors (FIGURE 4). $\Delta F_{-\text{CH}_2-}$, as obtained from the slope of the lines, -370 cal. per mole (for both systems); ΔF_{COO^-} , as obtained from the intercepts of the lines with the ordinate axes (at $-\text{CH}_2- = 0$), is -1650 cal. per mole with ferricyanide as acceptor, and -880 cal. per mole with cytochrome c as acceptor.

The results suggest that ferricyanide increases the binding of the carboxylic end of fatty acids to the substrate site, possibly by the formation of an enzyme-inhibitor-ferricyanide complex; it has been proposed that a ternary enzyme-substrate-acceptor complex is formed in this system (Morton *et al.*, 1961).

Also D-lactate dehydrogenase of anaerobic yeast is inhibited by fatty acids, the effect increasing with the number of $-\text{CH}_2-$ groups of the inhibitor molecules. The inhibition is not fully competitive, however, and for this reason study of the type described above is not possible.

References

- APPLEBY, C. A. & R. K. MORTON. 1954. Crystalline cytochrome b_2 and lactic dehydrogenase of yeast. *Nature*. **173**: 749-752.
- ARMSTRONG, J. McD., J. H. COATES & R. K. MORTON. 1960. Flavin dissociation and inactivation of cytochrome b_2 by oxygen. *Nature*. **186**: 4-7.
- AUDRAS, A., M. IWATSUBO & F. LABEYRIE. 1960. Groupes flaviniques des lactico-déshydrogénases de la levure. *Comp. rend.* **250**: 2621-2623.
- BERGMANN, F. 1928. The specificity of dehydrogenases. *Biochem. J.* **22**: 1178-1192.
- BERGER, E., T. CREMONA & T. P. SINGER. 1960. The D- α -hydroxy acid dehydrogenase of yeast. *Biochem. and Biophys. Research. Com.* **2**: 298-302.
- BUDEL, A., L. NASLIN & F. LABEYRIE. 1959. Reactivation par le zinc de la D-lactico-déshydrogénase inhibée par l'acide éthylènediaminetetraacétique. *Compt. rend.* **249**: 1959-1961.
- CHATTERMAN, R. & P. P. SLONIMSKI. 1960. Effet différentiel des analogues structuraux d'aminoacides sur la formation des enzymes respiratoires induite par l'oxygène. *Comp. rend.* **250**: 220, 221.
- LABEYRIE, F., P. P. SLONIMSKI & L. NASLIN. 1959. Sur la différence de stéréospécificité entre la déshydrogénase lactique extraite de la levure anaérobie et celle extraite de la levure aérobie. *Biochim. et Biophys. Acta.* **34**: 262-265.
- ENDENMAYER, A. & L. SMITH. 1957. Some oxidative enzymes of anaerobically grown yeast. *Federation Proc.* **16**: 212.
- MORTON, R. K., J. McD. ARMSTRONG & C. A. APPLEBY. 1961. In *Proceedings of the Haematin Enzyme Symposium*. J. E. Falk, M. R. Lemberg & R. K. Morton, Eds. Pergamon Press. London, England.
- NYGAARD, A. P. 1961. The Enzymes. Vol. VII. P. D. Boyer, H. Lardy, and K. Myrbaeck, Eds. Academic Press. New York, N. Y.
- NYGAARD, A. P. 1958. Purification and properties of yeast lactic dehydrogenase. *Biochim. et Biophys. Acta.* **30**: 450.
- NYGAARD, A. P. 1959. Lactic dehydrogenase of yeast. I. Preparation and reaction properties. *Biochim. et Biophys. Acta.* **33**: 518-521.
- NYGAARD, A. P. 1959. Lactic dehydrogenase of yeast. II. Different forms of the enzyme. *Biochim. et Biophys. Acta.* **35**: 212-216.
- NYGAARD, A. P. 1960. Lactic dehydrogenase of yeast. III. A comparative study of the kinetic properties and the stability of two isolated forms of the enzyme. *Biochim. et Biophys. Acta.* **40**: 85-92.
- NYGAARD, A. P. 1961a. D-Lactic cytochrome c reductase, a flavoprotein from yeast. *J. Biol. Chem.* **236**.
- NYGAARD, A. P. 1961b. Induction of D- and L-lactate cytochrome c reductase in yeast. *J. Biol. Chem.* In press.
- NYGAARD, A. P. 1961c. Kinetic properties of yeast D-lactate cytochrome c reductase. *J. Biol. Chem.* In press.
- NYGAARD, A. P. 1961d. Reversible interaction of inhibitors at the active sites of D- and L-lactic cytochrome c reductase. *J. Biol. Chem.* In press.

THE FORMATION AND ENZYMATIC PROPERTIES OF THE VARIOUS LACTIC DEHYDROGENASES OF YEAST*

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Introduction

Since the studies of Meyerhof¹ in 1919 it has been known that yeast contains a lactic dehydrogenase that differs from the familiar lactic dehydrogenase of animal tissues in that it functions without added coenzymes. Between its discovery and eventual isolation in pure form, about 35 years later by Appleby and Morton² in Australia, and Boeri and his colleagues³ in Italy, many illustrious biochemists studied the properties of this enzyme or attempted its isolation⁴⁻⁸ without realizing that yeast contains not one but several lactic dehydrogenases that may be readily distinguished from each other.

Recognition of the fact that yeast contains two or more lactic dehydrogenases besides the well-known hemoflavoprotein just discussed (to which we shall refer as b_2 in this paper, since cytochrome b_2 is an essential constituent part of it) came about in a curious, indirect way. It has been known for many years (cf. Slonimski⁹) that most of the enzyme systems present in the mitochondria of animal tissues, including those responsible for the Krebs cycle and electron transport, were absent in anaerobic yeast and were formed gradually as the cell adapted itself to oxygen. In aerobically grown cells these enzymes were always present, and appeared to be associated with structural elements of the cell, which some investigators believed to be not only functionally but also structurally analogous to mitochondria.⁹⁻¹² While now it appears more likely that the structures referred to are not pre-existing mitochondria but fragments of the cell membrane formed during mechanical disintegration of the cell, it remains true that these fragments—and hence the parent membrane—are the loci of the respiratory activities of the yeast cell and are functionally analogous to mitochondria.

Among the components absent in anaerobic yeast are most of the normal cytochromes of aerobic cells, including cytochrome b_2 , the hemoprotein associated with L-lactic dehydrogenase. In fact, using accepted methods of assay of this enzyme, which involve the measurement of the rates of reduction of cytochrome c or of methylene blue by lactate, Slonimski⁹ could not detect any activity in anaerobic cells, and the activity rapidly rose during O_2 adapta-

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† On leave of absence from the University of Padua, Padua, Italy.

‡ Fellow of the John Simon Guggenheim Memorial Foundation, New York, N.Y., during the first phase of this study (October, 1959, to February, 1960).

§ Deceased, October 28, 1960.

ion. Thus the situation appeared to be quite analogous to that of succinic dehydrogenase, which had been claimed to be absent in anaerobic yeast and to appear very rapidly during subsequent adaptation to O_2 .⁹

About four years ago, Hebb *et al.*¹³ in our Detroit laboratory reinvestigated the question of the absence of succinic dehydrogenase in anaerobic yeast. Recognizing that the methods used by earlier workers would be incapable of detecting yeast succinic dehydrogenase, even if it were present, they employed the dye phenazine methosulfate as electron acceptor, which provides the only known satisfactory method for the assay of this enzyme in yeast. They found that succinic dehydrogenase was far from absent in anaerobic cells, although its concentration was somewhat lower than in aerobic yeast, but that the members of the respiratory chain were completely absent in anaerobic yeast and, since these were required for the operation of the assay method of Slonimski, he had been unable to detect the enzyme.

Although cytochrome *c* and methylene blue are satisfactory electron acceptors for purified L-lactic dehydrogenase (b_2), there were claims in the literature, mostly from Okunuki and his colleagues in Osaka, Japan,^{14,15} that this enzyme may exist in forms that cannot reduce methylene blue or cytochrome *c* but could react well with other oxidants. Recalling that in the case of succinic dehydrogenase the proper choice of the assay was the clue to establishing the presence of the enzyme in anaerobic yeast, the Detroit group decided to reinvestigate the absence of the enzyme in anaerobic cells, using ferricyanide as an oxidant.¹⁶ It was soon found that DL-lactate was even more rapidly oxidized in membrane fragments isolated from anaerobic than from aerobic yeast with ferricyanide as the acceptor, but with cytochrome *c* only aerobic cells oxidized lactate at appreciable rates. By means of the ferricyanide technique several other groups independently established the oxidation of DL-lactate by anaerobic cells (Slonimski and Tysarowski¹⁷ in France; Boeri *et al.*¹⁸ in Italy; and Lindenmayer and Smith¹⁹ in the United States). Thus the situation seemed superficially analogous to succinic dehydrogenase and was, in fact, so interpreted by Slonimski and Tysarowski in their early papers,^{17,20} which suggested that anaerobic yeast contains a precursor of the enzyme present in aerobic cells. Later Labeyrie and her colleagues²¹ discovered that the "lactic dehydrogenase" of anaerobic cells is specific for the D configuration of lactate, while the enzyme isolated from aerobic cells (and then thought to be the only one present) had been known to be specific for L-lactate.³ This difference in stereospecificity and differences in kinetic constants led Labeyrie *et al.*²¹ to the conclusion that one enzyme could not be the precursor of the other.

While the precursor-product hypothesis thus appeared to be dead and buried, it has been exhumed lately by Slonimski's group,²² and has also been revived by Nygaard.^{23,24}

Because of the intrinsic interest of the problem and its implications on enzyme-forming processes, in late 1959 the laboratories in Ferrara, Italy, and in Detroit decided to pool their efforts in the form of a four months' collaboration in order to settle the question of the relation of the D and L enzymes. In the course of this collaborative research program, evidence was obtained indicating that these two enzymes enjoy separate and independent existence and that a

precursor-product relationship between them is extremely unlikely. A brief summary of the relevant findings will be given later in this paper. Incidental to this investigation was the finding that yeast cells contain at least one and probably two additional, hitherto unrecognized enzymes for the oxidation of lactate. The main part of this paper will deal with the evidence for the existence of the various lactic dehydrogenases of yeast, and will review what is known of their chemical identity, the conditions necessary for their formation, and their probable interrelations.

Enzymes for the Oxidation of D(-) Lactate

Thus far two well-defined enzymes capable of oxidizing D-lactate to pyruvate have been isolated from yeast: the enzyme from anaerobic yeast (Slonimski *et al.*¹⁷ and Cremona and Boeri, in preparation), which has been shown by Boeri *et al.*²⁵ to be a general D- α -hydroxy acid dehydrogenase, and an enzyme present in aerobic yeast, discovered in our collaborative work in Ferrara²⁶ and more recently purified in our laboratory in Detroit,²⁷ as well as by Nygaard,²⁸ to which we shall refer as D-lactic cytochrome reductase. While these two enzymes are strikingly similar in some respects, they are readily distinguishable from each other.

As already mentioned in the introduction, the first of these enzymes is formed in largest amounts during anaerobic conditions, and it has been reported^{17,21} to decline rapidly on incubation of the cells in the presence of O₂ and in the absence of active cell division (that is, under conditions of the so-called O₂ adaptation), conditions that also happen to evoke the formation of cytochrome *b*₂.⁹ It was precisely for these reasons that first Slonimski and his colleagues and, later, Nygaard were led to believe that the D- α -hydroxy acid dehydrogenase is a precursor of the *b*₂. As shown in TABLE 1, however, the decline in D- α -hydroxy acid dehydrogenase activity is just as great under absolutely anaerobic conditions as in O₂. In fact, in a previous publication²⁶ we have demonstrated that this enzyme is formed only during the rapid phase of cell division and is rather rapidly destroyed in living cells once active cell division stops. That its disappearance on incubation of the cells in the absence of a nitrogen source has nothing to do with the formation of *b*₂ is further shown by the facts (1) that conditions may be readily established under which the decline of D- α -hydroxy acid dehydrogenase is unaccompanied by any synthesis of *b*₂, and (2) that, conversely, the conditions of adaptation may be so adjusted that rapid synthesis of *b*₂ occurs, without a significant decline in D- α -hydroxy acid dehydrogenase activity, as shown in experiment 2 of TABLE 1.

Some of the known similarities and differences between the two D-specific enzymes that have been isolated and characterized are summarized in the last two columns of TABLE 2. While the general D- α -hydroxy acid dehydrogenase is characteristically associated with young, anaerobic cells, the D-lactic cytochrome reductase is an enzyme typical of aerobic cells. It is also formed during "O₂ adaptation" of anaerobic yeast, although not at the same rate as *b*₂.

Both enzymes appear to require a divalent metal for activity and both are probably flavoproteins. The flavoprotein nature of the D- α -hydroxy acid dehydrogenase was first suggested in 1958 by Boeri *et al.*¹⁸ and by Slonimski and

Cysarowski,¹⁷ who also considered it to be a flavin adenine dinucleotide (FAD) enzyme; however, to our knowledge, the identity of the flavin has not been critically demonstrated, and even the flavoprotein nature of the enzyme has only recently been critically established by Boeri *et al.*²⁵ FIGURE 1, taken from their work, shows the difference spectrum obtained on addition of D(-)lactate to a purified preparation, and clearly shows bands at 450 and 380 $m\mu$. The flavoprotein nature of the cytochrome reductase has been demonstrated in unpublished experiments in this laboratory by resolving it with ammonium sulfate and reconstituting it with authentic FAD.

TABLE 1

D- α -OH ACID DEHYDROGENASE AND CYTOCHROME B_2 ACTIVITIES OF ANAEROBICALLY GROWN YEAST AFTER VARIOUS CONDITIONS OF ADAPTATION*

Experiment	Conditions	O ₂	μ Moles lactate oxidized/min.			
			D(-)lactate— K ₃ Fe(CN) ₆		L(+)lactate— cyt. c	
			Per ml.	Per mg.†	Per ml.	Per mg.†
(1)	"Grandes"		32	0.62		
	Same, adapted 6 hrs. in O ₂		0	0		
	Same, adapted 6 hrs. in N ₂		0	0		
(2)	"Grandes"	<10	12.6	0.23	0	0
	Same, adapted 4 hrs. in air	22	13.9		0.37	
	Same, adapted 22.5 hrs. in air	53	11.3	0.2	3.81	0.071

* Adaptation means incubation in stationary conditions.

† Activity per milligram biuret protein.

Yeasts were grown anaerobically in 10 per cent glucose, 1 per cent yeast extract, and 1 per cent peptone. Adaptations were performed with thoroughly washed cells with constant bubbling with the gas indicated at 30° C. in the following media: experiment 1, 0.066 *M* KH₂PO₄—10 per cent glucose; experiment 2, 0.066 *M* KH₂PO₄—7 mM glucose—54 mM DL-lactate.

Incubation with or dialysis against versene or *o*-phenanthroline completely inactivates both enzymes.^{25,27,32} Incubation with extremely low amounts of certain divalent metals, under suitable conditions, reactivates both. Co⁺⁺, Zn⁺⁺, Mn⁺⁺, and Fe⁺⁺, to varying extents, reactive both enzymes.^{25,32a} Perhaps the most striking similarity between these two dehydrogenases is that the function of the metal in both appears to be substrate binding, as will be discussed later.

As to substrate specificity, the D- α -hydroxy acid dehydrogenase oxidizes a wide range of D- α -OH acids, besides D(-)lactate, including α -OH butyrate, malate, α -OH isobutyrate, α -OH caproate, α -OH isocaproate, α -OH isovalerate, and glycerate, but it is inactive with L-hydroxy acids, β -OH butyrate, hydroxy-amino acids, and mandelic acid (TABLE 3). In contrast, the D-lactic cytochrome reductase has a more restricted specificity, and fails to oxidize a number of

substances that are excellent substrates for the D- α -hydroxy acid dehydrogenase (TABLE 4). The Michaelis constants of the two enzymes for the same substrates are also quite different (TABLE 2). Oxalate is a very powerful competitive inhibitor of the first enzyme and probably acts by competing with the substrate

TABLE 2
COMPARISON OF PROPERTIES OF L-LACTIC DEHYDROGENASE,* D- α -OH ACID DEHYDROGENASE, AND OF D-LACTIC-CYTOCHROME REDUCTASE

Property	L-lactic dehydrogenase	D- α -OH acid dehydrogenase	D-lactic-cytochrome reductase
Prosthetic groups	Protohaem, FMN	Divalent metal and FAD	Divalent metal and FAD
Effect of dialysis against metal chelators	None	Inactivates	Inactivates
Metals that reactivate the enzyme after treatment with metal chelators	None	Co ⁺⁺ , Zn ⁺⁺ , Mn ⁺⁺ , Fe ⁺⁺	Co ⁺⁺ , Zn ⁺⁺ , Mn ⁺⁺ , Fe ⁺⁺
Substrates	L(+)lactate, L- α -OH butyrate, glycolate	Many D- α -OH acids	D(-)lactate, D- α -OH butyrate
K _M for lactate at 30° C.	$8 \times 10^{-4} M$	$5.5 \times 10^{-4} M$	$2.85 \times 10^{-4} M$
K _M for α -OH butyrate		$4 \times 10^{-3} M$	$1.42 \times 10^{-3} M$
Effect of oxalate	None	Inhibitory at very low concentration	Inhibitory at moderate concentration
Competitive inhibitors		Oxalate, malonate, tartronate, α -keto-glutarate, L-malate, L(+)lactate	
pH optimum (lactate)	8	8	7.5
Stability at pH of optimum stability	Moderately stable anaerobically	Extremely unstable	Very stable
Oxidants	Fe(CN) ₆ ⁺⁺⁺ , cytochrome <i>c</i> , phenazine methosulfate, methylene blue, O ₂ , and others	Fe(CN) ₆ ⁺⁺⁺ , menadione, methylene blue, DCPIP	Cytochrome <i>c</i> , phenazine methosulfate
Extractability	Solubilized by butanol or autolysis	Easily solubilized, apparently cytoplasmic	Very difficultly solubilized, particle bound
pH of optimum stability	6.5 to 7.8		6.5 to 7.5

* Cytochrome *b*₂.

The data on L-lactic dehydrogenase are based on the papers of Appleby and Morton,^{29,30} and Boeri *et al.*^{3,31}; those on D- α -OH acid dehydrogenase are from Boeri *et al.*,²⁵ or are unpublished data of T. Cremona; the data on D-lactic cytochrome reductase are adapted from Gregolin and Singer,²⁷ or are unpublished data of C. Gregolin.

for the metal component; the second enzyme is inhibited only at moderately higher concentrations of oxalate.²⁶ The same is true of a number of other competitive inhibitors: for example, the D- α -hydroxy acid dehydrogenase is much more sensitive to inhibition by L-malate than the D-lactic cytochrome reductase. The pH optima (with lactate as substrate) differ slightly, but the pH stability curves are very different. While the cytochrome reductase is

quite stable around pH 6.5, and is rapidly inactivated at pH 8, the D- α -hydroxy acid dehydrogenase is more stable at pH 8 than at pH values below 7 and is, in general, much more unstable to a variety of treatments than the cytochrome reductase.

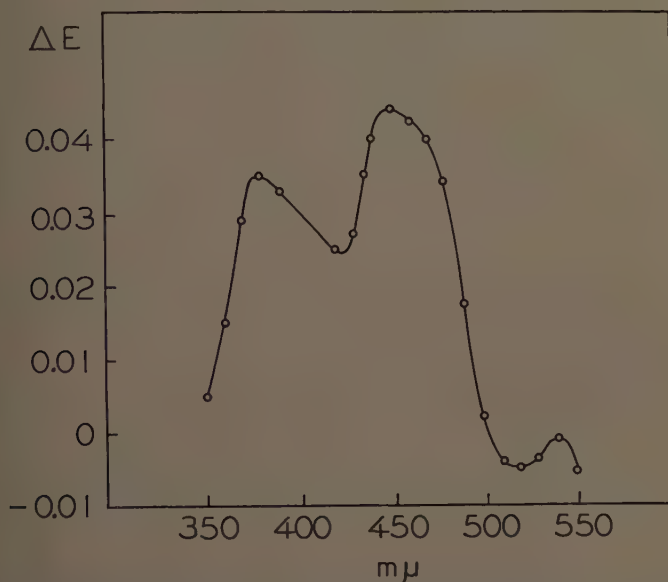


FIGURE 1. Difference spectrum of D- α -hydroxy acid dehydrogenase (oxidized minus reduced by D(-)lactate). From Cremona and Boeri (in preparation).

TABLE 3
SUBSTRATE SPECIFICITY AND K_M VALUES OF D- α -OH ACID DEHYDROGENASE*

Substrate	K_M	Relative rate ($V_{max.}$)
D-Lactate	$5.5 \times 10^{-4} M$	1.00
D-Malate	$3.7 \times 10^{-3} M$	0.89
DL- α -Hydroxybutyrate	$4 \times 10^{-3} M$	1.08
DL- α -Hydroxycaproate	$6.5 \times 10^{-4} M$	1.00
DL- α -Isocaproate	$4.25 \times 10^{-4} M$	0.79
Glycerate		0.019

* At 30° C. pH .

Adapted from Boeri *et al.*²⁵ and Ghiretti-Magaldi *et al.*^{32a}

As to electron acceptors, the specificity of the reductase is much more restricted than that of the D- α -hydroxy acid dehydrogenase. In fact, among the oxidants tested, only cytochrome *c* and phenazine methosulfate are active (TABLE 5). The latter is capable of measuring far greater rates at infinite concentration of acceptor than the former. In view of the inactivity of dichlorophenolindophenol, a convenient spectrophotometric test consists of using phenazine methosulfate as a carrier, the indophenol dye as the terminal oxidant, and measuring the rate of decolorization of the latter. While cytochrome *c* and

phenazine methosulfate do not react with the D- α -hydroxy acid dehydrogenase, all the dyes that were found to be inactive with the reductase work well with this enzyme (TABLE 6).

It might be well to mention at this point the fact that the natural electron acceptor of neither D enzyme is known at present. The fact that cytochrome *c* is a good acceptor for the reductase does not mean, of course, that a direct interaction with cytochrome *c* occurs in the cell. Although the localization of the enzyme in the mitochondrial membrane, where the electron transport chain

TABLE 4
SUBSTRATE SPECIFICITY OF D-LACTIC-CYTOCHROME REDUCTASE

Substrate	Activity relative to D(-)lactate at V_{max} . (30° C.)	K_M at 30° C.
D-Lactate	1	$2.85 \times 10^{-4} M$
D- α -Hydroxybutyrate	0.41*	$1.4 \times 10^{-3} M^*$
D-Malate	0	
DL- α -OH isobutyrate	0	
DL- α -OH caproate	0	
DL- α -OH isocaproate	0	
DL- α -OH isovalerate	0	

* Assuming no inhibition by L-antipode: tests were run with DL- α -OH butyrate. Assays at pH 7.5, 30° C., in 0.05 *M* imidazole with cytochrome *c* as acceptor, using a purified enzyme. Adapted from Gregolin and Singer²⁷ and unpublished data of C. Gregolin.

TABLE 5
SPECIFICITY OF D-LACTIC CYTOCHROME REDUCTASE FOR ELECTRON ACCEPTORS

Oxidant	Relative activity at infinite concentration (V_{max} .)
Cytochrome <i>c</i>	1
Phenazine methosulfate*	8.4
Menadione†	0
Ferricyanide	0
2,6-DCPIP	0

* Measured spectrophotometrically with DCPIP as terminal acceptor.

† Measured spectrophotometrically with DCPIP or ferricyanide as terminal acceptor. Adapted from Gregolin and Singer²⁷ and unpublished data of C. Gregolin.

is also located, is compatible with the idea that the enzyme might interact with the respiratory chain, the following experiment clearly shows that D-lactic cytochrome reductase is not a respiratory chain-bound enzyme in yeast.²⁷ Freshly isolated particles, with very high respiratory activity, were used to compare the oxidation of D-lactate with that of succinate, a typical respiratory chain-linked substrate, using a rotating platinum microelectrode. The oxidation of succinate, whether or not stimulated by the addition of external cytochrome *c*, was 100 per cent antimycin-sensitive. D-Lactate alone was not at all oxidized by the preparation unless cytochrome *c* was also added; that is the enzyme could not transfer electrons to the bound cytochrome *c* of the particles. When external cytochrome *c* was added, the oxidation of D-lactate immediately began, but the resulting O₂ uptake was virtually unaffected by antimycin.

Thus cytochrome *c* in this case acts as a dye, bypassing the respiratory chain up to cytochrome oxidase.

This brings us to the last point of dissimilarity between the two enzymes, and a very important one. The D- α -hydroxy acid dehydrogenase is readily and completely liberated in solution by autolysis or by mechanical breakage with Ballotini beads, and all available evidence points to the conclusion that it is a cytoplasmic enzyme (TABLE 2). In contrast the reductase is particle-bound,²⁷ as has been found also by Nygaard.^{24,28} Only a very small per cent of the enzyme is liberated by autolysis of dried yeast.²⁶ Advantage can be taken of the strong attachment of the enzyme to membrane fragments in its purification. As in the case of the succinic dehydrogenase of yeast,³³ major purification is accomplished by the preliminary isolation of the membrane fragments (the so-called "mitochondria") by differential centrifugation. Extraction of the enzyme was first attempted by mild enzymatic procedures, which usually liberate particle-bound enzymes in soluble form, such as digestion of the particles with phospholipase A, with a purified fungal lipase of very broad specificity,³⁴ and

TABLE 6
SPECIFICITY OF D- α -OH ACID DEHYDROGENASE FOR ELECTRON ACCEPTORS

Active	Inactive
Menadione*	Phenazine methosulfate*
K ₃ Fe(CN) ₆	Cytochrome <i>c</i>
Methylene blue*	O ₂
2,6-DCPIP*	1,4-napthoquinone-5-sulfonate*

* Adapted from Slonimski and Tysarowski,¹⁷ Cremona and Boeri (unpublished); and Boeri *et al.*²⁵

with a concentrate of the autolyzing enzymes of yeast, in each case with negative results. Treatment of acetone powders of the particles with mildly alkaline buffers failed to extract the enzyme at *pH* values where it was stable. As the *pH* of the extractant was increased, more and more of the protein was extracted, but the inactivation also increased with *pH* and, thus, the protein extracted at more alkaline *pH* values was largely inactivated (FIGURE 2). A method elaborated in this laboratory a few years ago for the extraction of succinic dehydrogenase of yeast gave somewhat more promising results: it involves the desiccation of fresh particles or of acetone powders thereof with *n*-butanol and ether, followed by extraction with mildly alkaline buffers.³³ This method, which also appears to have been used by Nygaard,²⁸ also results in progressive solubilization of the enzyme as the *pH* of the buffer used is increased. Unfortunately, however, more and more inactivation again occurs as the *pH* is increased above about *pH* 8 at 0° C. Thus, while at more alkaline *pH* values all or most of the protein might be extracted, it would be largely a denatured product. At *pH* 7.8, the most alkaline *pH* permissible under the experimental conditions, only about 18 per cent of the activity is extractable. Extraction with aqueous butanol (Morton's procedure)³⁵ gave essentially negative results. Since the yield in the butanol process is so low, we have elaborated an alternative method that involves extraction of the particles with 4 per cent (w/v)

Triton X-100, a neutral nonionic detergent. Although we were well aware of the fact that, despite recurrent claims to the contrary, extraction with detergents such as cholate, deoxycholate, and even nonionic ones probably never yields enzymes in true solution but only in dispersion and that the purification

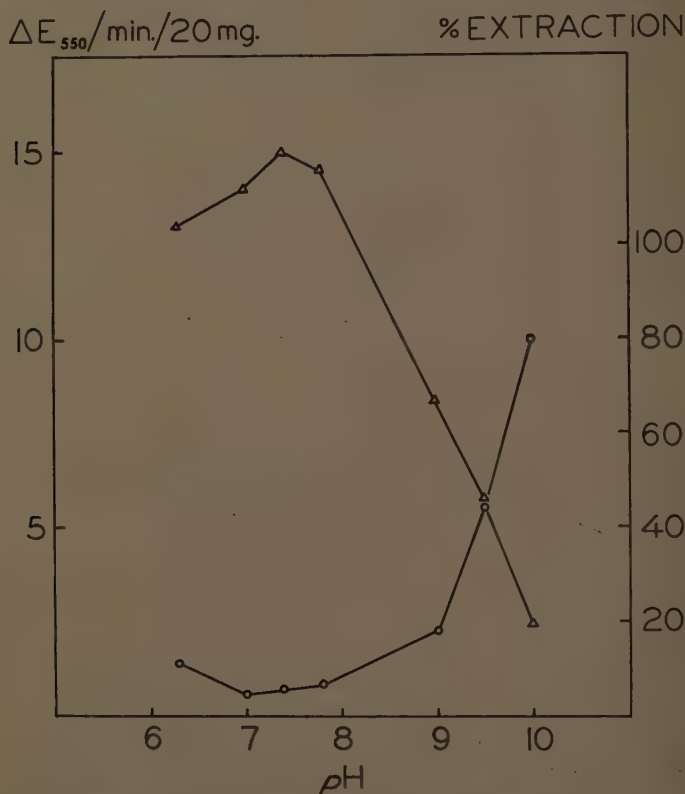


FIGURE 2. Effect of pH on the inactivation and on solubilization of D-lactic cytochrome reductase from acetone powders of yeast particles. A 2 per cent (w/v) suspension of acetone powder in 0.1 M buffer was homogenized and stirred for 10 min. at 0° C. at the pH values indicated, then briefly centrifuged at high speed. Both the suspension before centrifugation and the supernatant solution were assayed at pH 7.5 in the standard assay.²⁷ The buffers employed were triethanolamine (up to pH 7.8), Tris (pH 9), and glycine (pH 9.5 to 10). The pH values on the abscissa refer to 0° C.; activity is expressed per 20-mg. acetone powder on the left ordinate and the per cent extraction (activity of supernatant solution/activity of uncentrifuged suspension) is noted on the right ordinate. Circles, per cent extraction; triangles, activity in suspension before centrifugation. Unpublished data of Gregolin and Singer.

of such extracts is often difficult and sometimes impossible, we decided to purify the Triton-extracted enzyme, since the yield was much better than in the butanol procedure and since it readily led to preparations of high specific activity (TABLE 7). Much of our work on the function of the metal in this enzyme has been performed with this type of preparation.*

* More recently it was found that after partial purification of Triton extracts digestion with phospholipase A yields true solutions of the enzyme. The resulting solution has been purified 600-fold, compared with the parent particles.

Before we leave the subject of the comparison of the two D-lactic enzymes, the most striking similarity between them, the behavior of their metal constituent, deserves some comment. Although the mode of action of the metal in these enzymes is of considerable theoretical interest, since these may in fact be the first metal flavoproteins in which the function of the metal is amenable to direct experimental study at this time,³⁶ the subject is too specialized to be treated *in extenso* in this publication. Thus only a brief summary of the findings will be presented here.

Treatment of either enzyme with metal chelating agents, such as versene or *o*-phenanthroline, results in inactivation; delayed addition of suitable divalent metals reactivates either enzyme to varying extents, depending on experimental

TABLE 7
SUMMARY OF PURIFICATION OF D-LACTIC-CYTOCHROME REDUCTASE

Step	Total activity μM lactate/min.	Specific activity μM lactate/min./mg.
Yeast particles	180	0.060
Acetone powder of yeast particles	175	0.055
Triton extract	183	0.546
Eluate from calcium phosphate gel	161	1.80

Activities are measured at pH 7.5, 30° C., in imidazole buffer, with cytochrome *c* as acceptor. The activity in the phenazine methosulfate assay²⁷ is 8.4 times higher. Adapted from unpublished data of C. Gregolin.

TABLE 8
METALS CAPABLE OF REACTIVATING D- α -OH ACID DEHYDROGENASE AND D-LACTIC CYTOCHROME REDUCTASE FOLLOWING DIALYSIS AGAINST VERSENE OR *o*-PHENANTHROLINE AND PASSAGE THROUGH SEPHADEX

Enzyme	Metals active	Metals inactive
D- α -OH acid dehydrogenase	Zn ⁺⁺ , Co ⁺⁺ , Mn ⁺⁺ , Fe ⁺⁺	Mg ⁺⁺ , Ca ⁺⁺
D-Lactic cytochrome reductase	Zn ⁺⁺ , Co ⁺⁺ , Mn ⁺⁺ , Fe ⁺⁺	

Adapted from data of Ghiretti-Magaldi *et al.*^{32a}

conditions.^{25,32,32a} Since the combination of the enzyme with the chelating agent appears to be a relatively slow process (at least with the reductase), in practice overnight dialysis against a chelator is employed, followed by dialysis against a suitable buffer and, finally, passage through a column of Sephadex gel.^{25,32a} Both of the latter treatments are designed to remove unbound chelator. The resulting enzyme is reactivated by very low concentrations of certain divalent metals (TABLE 8). FIGURE 3 is an example of the reactivation of D- α -hydroxy acid dehydrogenase. FIGURES 4 and 5 illustrate the reactivation of the reductase and further demonstrate that the extent of reactivation depends on the nature of the substrate (FIGURE 5) and that, with a given substrate, different metals give different maximal reactivation (V_{max}) and different *apparent* K_M values.

There is reason to believe that under the conditions of these experiments the reversible inactivation proceeds by two mechanisms (Scheme 1): in part the loss

of activity is due to the presence of bound chelator on the enzyme, which is not removed by dialysis or gel exclusion, and its reactivation by metals represents a displacement of the chelator by the added metal; in part it seems to represent a genuine resolution of holoenzyme into metal-free flavoprotein, which is inactive but may be reactivated by a suitable metal. In fact, with both enzymes, loss of the metal and consequent reversible inactivation may occur

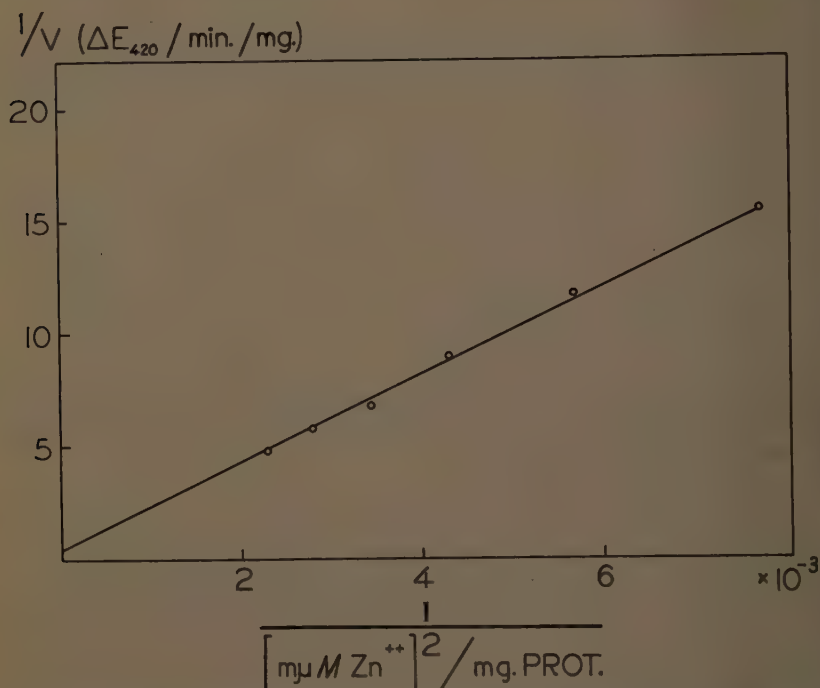


FIGURE 3. Reactivation of D- α -hydroxy acid dehydrogenase by Zn^{++} . A purified preparation was dialyzed for 12 hours at pH 7.4, 0°C ., under N_2 against $7 \times 10^{-3} M \text{Na}_2\text{HPO}_4$, $3 \times 10^{-3} M$ thioglycollate and $4 \times 10^{-3} M$ versene; then it was dialyzed for 3 hours against $0.01 M$ Tris buffer, pH 7.4 and, finally, passed through a column of Sephadex G-25, equilibrated with $0.01 M$ Tris buffer, pH 7.4. Samples were preincubated for 10 min. at 0°C . with varying amounts of Zn^{++} and assayed under standard conditions²⁵ in the ferricyanide assay. A plot of $1/V$ against $1/\text{Zn}$ or $1/\text{Zn}/\text{mg. protein}$ gave irrational values, but a rational curve was obtained by the method shown. From Ghiretti-Magaldi (in preparation).

"spontaneously" under rather mild conditions of purification (Cremona and Boeri; Ghiretti-Magaldi; both in preparation). This behavior may be one of the causes of the extreme instability of this enzyme, which has seriously hampered efforts to isolate it in many laboratories.

Besides the *reversible* inactivations just discussed, under conditions of prolonged dialysis both enzymes seem to undergo an irreversible inactivation, which might indicate that the loss of the metal may also lead to secondary denaturation; that is, that one function of the metal might be to stabilize the structure of these proteins.

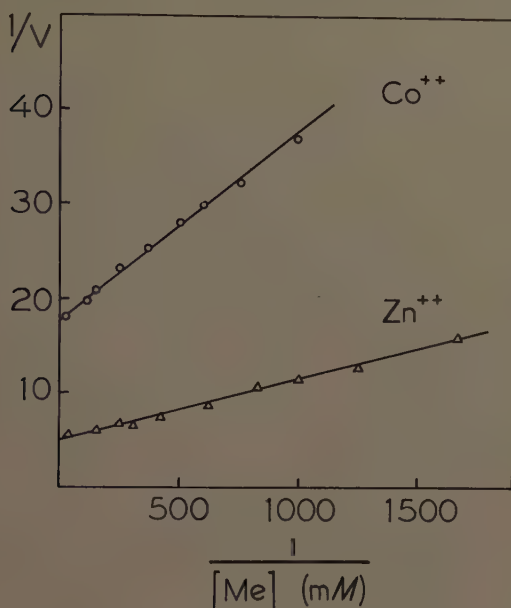


FIGURE 4. Reactivation of D-lactic cytochrome reductase. Inactivation was performed as in FIGURE 3, except that 0.01 *M* phosphate buffer, pH 6.5, containing 3.5×10^{-3} *M* *o*-phenanthroline was used in the first dialysis. Preincubation of the dialyzed enzyme with metals was performed at pH 6.5, 0° C., for 10 min. Activity is expressed as $1/\Delta E_{550}$ per min. per 0.075 mg. protein in the standard assay.²⁷ The metal concentration refers to that existing in the final assay mixture in a 3 ml. total volume. From Ghiretti-Magaldi (in preparation).

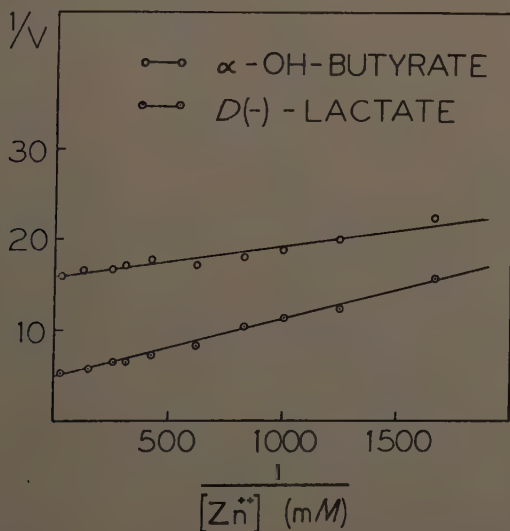


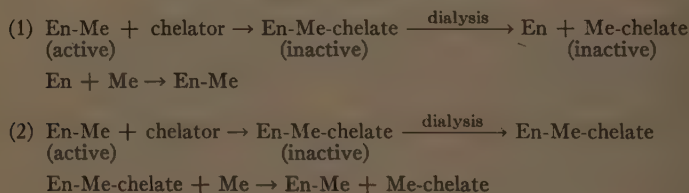
FIGURE 5. Reactivation of the reductase by Zn as tested with different substrates. Inactivation as in FIGURE 4. Excess substrate (30 μ moles D-lactate or DL- α -OH butyrate per 3 ml. final volume. Other conditions as in FIGURE 4. From Ghiretti-Magaldi (in preparation).

Despite remaining uncertainties about the mechanism of the reversible changes that the metal components of these enzymes undergo, the most probable interpretation of all available data is that in both enzymes the metal serves as the binding site of the substrate. A similar conclusion has been independently reached by Tubbs³⁷ regarding the function of the postulated metal moiety of the D- α -hydroxy acid dehydrogenase of kidney mitochondria. As is also true of both enzymes from yeast, the identity of the metal component of the mammalian enzyme³⁷ remains to be established.

Before leaving the subject of the D-lactic enzymes, it may be mentioned that the two enzymes discussed may not be the only ones present in yeast. We have reported²⁶ that strictly aerobically grown yeast contains an enzyme for the oxidation of D-lactate by ferricyanide which, like the enzyme from anaerobic cells, is readily obtained in solution by autolysis. Compared with anaerobic cells its activity is low (about 10 to 15 per cent as much). Whether this activity represents a third, separate D-lactic enzyme, as Nygaard²⁸ believes, remains a subject for further investigation.

SCHEME 1

POSSIBLE MECHANISMS OF REACTIVATION OF D- α -HYDROXY ACID DEHYDROGENASE AND D-LACTIC CYTOCHROME REDUCTASE



Enzymes for the Oxidation of L-Lactate

Although the existence of many L-lactic dehydrogenases or of many forms of the enzyme from yeast has been claimed in the literature,^{15,38,39} only one, the hemoflavoprotein cytochrome b_2 , has been isolated and characterized.^{2,3} The properties of the enzyme are too well known to need detailed discussion here. Perhaps a reference to TABLE 2, first column, may suffice to emphasize the major differences from the D-enzymes just discussed. The enzyme is present only in aerobic cells; it oxidizes only L(+) lactate, L- α -hydroxybutyrate and, to a small extent, glycollate. Unlike the two well-defined D-enzymes, protein-bound metals are not present and are not needed for its activity.³⁰

As to various forms of the enzyme, the only clear-cut instance is its crystallization by Appleby and Morton²⁹ in a complex form with deoxyribose polynucleotide, whereas the preparation of Boeri *et al.*³ was devoid of DNA and was noncrystallizable. Later it was shown⁴⁰ that the DNA is necessary for the crystallizability but not for the activity of the enzyme; once it is removed, the enzyme can no longer be crystallized. The significance of this DNA component thus remains quite obscure.

Among other investigators our group²⁶ has described an L-lactic dehydrogenase in yeast that appeared to differ from b_2 , since it was extracted from

"petite" mutants. Several years ago Slonimski,⁹ in an extensive study of these mutants, demonstrated that they were unable to synthesize the usual complement of mitochondrial enzymes, including the cytochromes (except for cytochrome *c*). Although Slonimski⁹ reported that the lactic dehydrogenase activity of petite and normal cells was equal, he could not detect the absorption bands of cytochrome *b*₂ by spectroscopic methods either at room temperature or at liquid-air temperature (cf. Figure 10 in Slonimski⁹). In view of this fact and of the circumstance that Slonimski had employed DL-lactate in his assays, it appeared possible that he had been measuring the oxidation of D(-)lactate by one of the enzymes discussed. Since, however, in our experiments preparations from aerobically grown petite cells oxidized L(+)lactate at an appreciable rate with either ferricyanide or cytochrome *c* as the electron acceptor,²⁶ it appeared that either the mutants contain an L(+)lactic dehydrogenase different from *b*₂, or that the enzyme is identical with the *b*₂ of normal aerobic cells but was spectroscopically undetectable in crude preparations because of its presence

TABLE 9
OXIDATION OF L(+) LACTATE IN AEROBIC NORMAL YEAST AND IN PETITE MUTANTS

Method of extraction	Activity		Ratio: normal/petite
	Normal	Petite	
Autolysate of dried yeast	8 μ M/min./g* dried yeast	2.56 μ M/min./g*	3.1
Fresh, compressed yeast extracted by mechanical breakage	9.5–11.3 μ M/min./g fresh yeast†	2.7–2.9 μ M/min./g*	3.7 \pm 0.2

* Grown in laboratory under vigorous aeration at 27° C. in 1 per cent peptone—1 per cent yeast extract—10 per cent glucose.

† Commercial (Red Star) bakers' yeast. Adapted from data of C. Gregolin and A. Ghiretti-Magaldi.^{40a}

at appreciably lower concentration than in normal cells. In order to settle this question we decided to isolate the enzyme from petite cells and compare it with authentic cytochrome *b*₂.

TABLE 9 compares the rates of oxidation of L(+)lactate in aerobic normal and petite cells after extraction by autolysis or mechanical disintegration. By either technique about 30 per cent as much activity is found in petite as in normal yeast. The enzymes were then isolated from both normal and petite cells by Boeri and Rippa's⁴¹ simplified procedure that yields the pure enzyme in about a day's working time. Lactic dehydrogenase activity was followed in all fractions, and the enzymes for both types of cells behaved similarly during fractionation, if allowance is made for the much lower content in petite cells. The final product from the petite cells gave the typical spectrum of the hemolavoprotein *b*₂ (FIGURE 6), both in the oxidized form and after enzymic reduction. Definite proof that the L-lactic dehydrogenase of petite is indeed cytochrome *b*₂ is shown in TABLE 10. As seen in the last column, the ratio of the hemoprotein content to the lactic-dehydrogenase activity is the same in the enzyme isolated from the petite mutants and from normal yeast. Further

proof that all the enzymatic properties of the preparation are those of b_2 is presented in TABLE 11.

While these experiments dispose of the possibility of the existence of a lactic dehydrogenase different from b_2 in aerobic petites, there remain some puzzling

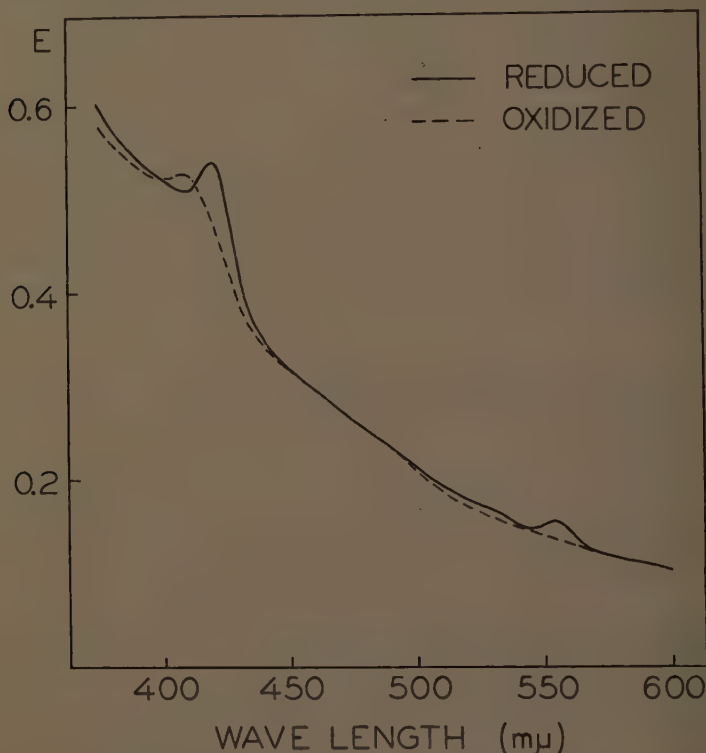


FIGURE 6. The enzyme was purified⁴¹ from petite yeast and freed from lactate and versene by gel exclusion. Residual cytochrome *c* was removed by passage through Amberlite IRC 50. The enzyme was then oxidized with a trace of $K_3Fe(CN)_6$, precipitated with 0.68 saturated $(NH_4)SO_4$, and resuspended to a final concentration of 30 mg./ml. The spectrum was taken with a Cary model 11 spectrophotometer at 25° C. in 1 ml. total volume. Immediately after the addition of 0.01 ml. 1 *M* DL-lactate the reduced spectrum was recorded.

TABLE 10
PROOF OF THE IDENTITY OF THE L(+) LACTIC DEHYDROGENASE
ISOLATED FROM PETITE YEAST WITH CYTOCHROME B_2

Source of enzyme	Activity (μM lactate oxidized per min. per ml.)	Cytochrome b_2 content ΔE_{557} (oxidized-reduced by lactate)	Ratio: activity ΔE_{557}
Normal yeast	12	0.024	500
Petite yeast, prep. 1	6.45	0.013	496
Petite yeast, prep. 2	9.75	0.019	511
Petite yeast, prep. 3	9.60	0.021	456

Adapted from data of C. Gregolin and A. Ghiretti-Magaldi.^{40a}

observations on the occasional appearance of an appreciable activity for L(+)-lactate in *anaerobically* grown petites (TABLE 12). The activity is obtained in autolysates and is measured with ferricyanide but is not detectable

TABLE 11
COMPARISON OF L(+)-LACTIC DEHYDROGENASES ISOLATED
FROM AEROBIC NORMAL AND PETITE YEASTS

Property	Normal	Petite
Conditions of formation	Aerobic growth or O ₂ -adaptation	Same
Substrates	Lactate, α -OH butyrate, and (glycollate)*	Lactate, α -OH butyrate (glycollate not tested)
Relative activity Fe(CN) ₆ /cyt. <i>c</i>	1†	1
Inhibitors	<i>p</i> -CMB, high oxalate	Same
Noninhibitory	CN ⁻ , versene	Same
<i>K_M</i> for L(+)-lactate	3.5×10^{-4} M at 20° C.*	8×10^{-4} M at 30° C.
pH optimum (Fe(CN) ₆ -assay)	8 at about 20° C.†	8 at 30° C.
Stability	Stabilized by substrate and anaerobic conditions	Same
Range of precipitation by acetone under specified conditions	12 to 40 per cent (v/v)	Same
Absorption maxima (after reduction by lactate)	423, 528, 557	423, 528, 557

* Adapted from Appleby & Morton (1959).³⁰

† Adapted from Boeri *et al.* (1955).⁸

TABLE 12
THE OXIDATION OF L(+)-LACTATE IN STRICTLY ANAEROBIC YEAST

Growth conditions	Rate of oxidation of L(+)-lactate in autolysates	
	μ M/min./ml.	μ M/min./mg. protein
Normal yeast, grown in 10 per cent glucose—1 per cent yeast extract—1 per cent peptone	0.38	0.007
Petite yeast, grown as above	0.81	0.017
Normal yeast, grown in Slonimski medium No. 3	0.36	0.0064
Petite, grown as above \pm DL-lactate present during growth	0.59	0.01

Assays at pH 8 in 0.08 M Tris at 30° C.; substrate = 5 mM L(+)-lactate; acceptor = 1 mM ferricyanide. Adapted from Singer *et al.*²⁶ and unpublished data from this laboratory.

with cytochrome *c*. Since cytochrome *c* is inactive as an oxidant and the enzyme is insensitive to 10^{-4} M *p*-chloromercuribenzoate and, since the cells were strictly anaerobic, it cannot be ascribed to the activity of cytochrome *b*₂. The insensitivity to mercurials and inactivity toward L-malate seem to preclude the involvement of D- α -hydroxy acid dehydrogenase plus a hypothetical race-nase. Since conditions to obtain it in a reproducible manner have not been

defined—in fact it is more often absent than present—we have not been able to undertake its characterization.

There have been other claims in the literature of the existence of L-lactic dehydrogenases with properties different from those of the hemoflavoproteins; but, in most instances, such claims were later withdrawn. Okunuki and his colleagues^{14,42} have separated the hemoprotein fragment of a crude preparation of b_2 by proteolysis, crystallized it, and shown that it was slowly reduced not only by lactate but also by malate and by TPNH in the presence of a crude yeast preparation. They suggested from this that b_2 is not a specific component of lactic dehydrogenase but a more general electron carrier, and that only the flavoprotein part could be considered as the lactic dehydrogenase. In a later paper⁴³ they appear to have recognized that the extremely slow rates of reduction of the hemoprotein part of the enzyme by yeast preparations has little relevance to normal physiological events and were ready to accept the thesis, widely held by others, that the cytochrome is part of the enzyme. As of this time, then, only one well-defined enzyme for the oxidation of L(+)-lactate has been isolated from yeast.

Concluding Remarks

In conclusion, while there are hints of the possible existence of additional lactic dehydrogenases in yeast, thus far only three have been definitely isolated and characterized. Since hitherto only gross differences have been studied, the existence of different molecular forms of these, detectable only by more refined techniques, also remains a possibility. There is no compelling evidence to believe that any two of these enzymes bear a precursor-product relationship to each other; an enzyme would not be expected to change substrate specificity in a radical fashion during the adaptation process. While precursors of these enzymes may and probably do exist, they are not expected to be endowed with catalytic activity and thus would not be detected by the techniques that have been applied.

References

1. MEYERHOF, O. 1919. *Pflügers Arch. ges. Physiol.* **175**: 20.
2. APPLEBY, C. A. & R. K. MORTON. 1954. *Nature*. **173**: 749.
3. BOERI, E., E. CUTOLO, M. LUZZATI & L. TOSI. 1955. *Arch. Biochem. Biophys.* **56**: 487.
4. BERNHEIM, F. 1928. *Biochem. J.* **22**: 1179.
5. OGSTON, F. J. & D. E. GREEN. 1935. *Biochem. J.* **29**: 1983.
6. ADLER, E. & M. MICHAELIS. 1935. *Z. Physiol. Chem.* **235**: 154.
7. DIXON, M. & L. G. ZERFAS. 1939. *Nature*. **143**: 557.
8. BACH, S. J., M. DIXON & L. G. ZERFAS. 1946. *Biochem. J.* **40**: 229.
9. SLONIMSKI, P. P. 1953. *La Formation des Enzymes Respiratoires chez la Levure*. Masson. Paris, France.
10. EPHRUSSI, B. & P. P. SLONIMSKI. 1955. *Nature*. **176**: 1207.
11. LINNANE, A. W. & J. L. STILL. 1955. *Arch. Biochem. Biophys.* **59**: 383.
12. EPHRUSSI, B. 1956. *Naturwissenschaften*. **43**(22): 505.
13. HEBB, C. R., J. SLEBODNIK, T. P. SINGER & P. BERNATH. 1959. *Arch. Biochem. Biophys.* **83**: 10.
14. OKUNUKI, K., B. HAGIHARA, I. SEKUZU & T. HORIO. 1958. *Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957*. : 264. Maruzen, Tokyo.
15. HORIO, T., J. YAMASHITA & K. OKUNUKI. 1959. *Biochim. et Biophys. Acta*. **32**: 593.
16. SINGER, T. P. & E. B. KEARNEY. 1959. *In Vitamin Metabolism. Proceedings of the Fourth International Congress of Biochemistry, Vienna, 1958. Symposium XI. O. Hoffman-Ostenhof, Ed.* : 209. Pergamon Press. London, England.

7. SLONIMSKI, P. P. & W. TYSAROWSKI. 1958. Compt. rend. acad. sci. **246**: 1111.
8. BOERI, E., E. CUTOLO & R. SACCOMANI. 1958. Bol. soc. ital. biol. sperim. **301**: 1887.
9. LINDENMAYER, A. & L. SMITH. 1957. Federation Proc. **16**: 212.
10. SLONIMSKI, P. P. 1958. Bull. Soc. Française Physiol. Végétale. **4**(4): 125.
11. LABEYRIE, F., P. P. SLONIMSKI & L. NASLIN. 1959. Biochim. et Biophys. Acta. **34**: 262.
12. KATTERMANN, R. & P. P. SLONIMSKI. 1960. Compt. rend. acad. sci. **250**: 220.
13. NYGAARD, A. P. 1960. Arch. Biochem. Biophys. **86**: 317.
14. NYGAARD, A. P. 1960. Arch. Biochem. Biophys. **88**: 178.
15. BOERI, E., T. CREMONA & T. P. SINGER. 1960. Biochem. Biophys. Research Comm. **2**: 298.
16. SINGER, T. P., E. B. KEARNEY, C. GREGOLIN, E. BOERI & M. RIPPA. 1960. Biochem. Biophys. Research Comm. **3**: 428.
17. GREGOLIN, C. & T. P. SINGER. 1961. Biochem. Biophys. Research Comm. **4**: 189.
18. NYGAARD, A. P. 1960. Preliminary report presented at the Third Scandinavian Summer meeting of Biochemistry, Medicinal Chemistry, Pharmacology, and Physiology. Oslo, Norway, August 1960.
19. APPLEBY, C. A. & R. K. MORTON. 1959. Biochem. J. **71**: 492.
20. APPLEBY, C. A. & R. K. MORTON. 1959. Biochem. J. **73**: 539.
21. BOERI, E. & L. TOSI. 1956. Arch. Biochem. Biophys. **60**: 463.
22. CURDEL, A., L. NASLIN & F. LABEYRIE. 1959. Compt. rend. acad. sci. **249**: 1959.
- 22a. GHIRETTI-MAGALDI, A., T. CREMONA, T. P. SINGER & P. BERNATH. 1961. Biochem. Biophys. Research Comm. In press.
23. SINGER, T. P., V. MASSEY & E. B. KEARNEY. 1957. Arch. Biochem. Biophys. **69**: 405.
24. YAMANAKA, T., T. HORIO & K. OKUNUKI. 1960. Biochim. et Biophys. Acta. **40**: 349.
25. MORTON, R. K. 1955. In Methods of Enzymology. S. P. Colowick and N. O. Kaplan, Eds. I: 25. Academic Press. New York, N.Y.
26. SINGER, T. P. & V. MASSEY. 1957. Record Chem. Progr. **18**: 201.
27. TUBBS, P. K. 1960. Biochem. Biophys. Research Comm. **3**: 513.
28. NYGAARD, A. P. 1959. Biochim. et Biophys. Acta. **33**: 517.
29. NYGAARD, A. P. 1959. Biochim. et Biophys. Acta. **35**: 212.
30. APPLEBY, C. A. & R. K. MORTON. 1960. Biochem. J. **75**: 258.
- 30a. GREGOLIN, C. & A. GHIRETTI-MAGALDI. 1961. Biochim. et Biophys. Acta. In press.
31. BOERI, E. & M. RIPPA. 1961. Arch. Biochem. and Biophys. In press.
32. YAMANAKA, T., T. HORIO & K. OKUNUKI. 1958. J. Biochem. (Japan). **45**: 291.
33. YAMASHITA, J., T. HORIO & K. OKUNUKI. 1958. J. Biochem. (Japan). **45**: 707.

MULTIPLE FORMS OF YEAST HEXOKINASE

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It has been known for some years now that certain enzymes, apparently homogeneous by a variety of criteria, can be separated into active fractions by column chromatography¹ or electrophoresis.² Despite the apparent differences in their molecular forms, there is in most cases very little difference in biochemical behavior between the multiple forms of a given enzyme.

Crystalline yeast hexokinase is separable into six active fractions by chromatography on DEAE cellulose or into three active fractions by electrophoresis on starch gel.³ In the present communication, studies on these multiple forms of hexokinase are described. By a modified procedure for chromatography on DEAE cellulose, two major fractions were obtained by pH gradient (Fraction I) and NaCl gradient elution (Fraction II). These fractions were identical with respect to their catalytic properties. Fraction I can be converted to Fraction II by treatment with trypsin or chymotrypsin.

Methods and Materials

The methods used for preparation and assay of the enzyme,⁴ column chromatography and starch gel electrophoresis,³ are as described by the authors cited unless otherwise specified.

For determination of the pH optimum, glucose disappearance was measured by the method of Hugget and Nixon.⁵ For assay of the ATP-ase activity of hexokinase, inorganic phosphate was measured according to Chen *et al.*⁶ after ATP was eliminated by charcoal adsorption.⁷ In an alternative assay for ATP-ase, ADP was determined by coupling the reactions of pyruvate kinase and lactic dehydrogenase⁸ using the assay mixture for pyruvate kinase of Bücher and Pfeleiderer,⁹ scaled down to a 1-ml. reaction mixture.

For isolation of a yeast culture from a single cell, commercial yeast was plated on heart infusion agar, and a single colony was picked for growth in a complete liquid medium at room temperature with aeration.

Results

Chromatography of crystalline enzyme. The separation of 6 times recrystallized yeast hexokinase into several fractions by DEAE-cellulose column chromatography is shown in FIGURE 1. At least 6 forms of hexokinase were separated, 3 major peaks (*b*, *e*, and *f*) and 3 minor peaks (*a*, *c*, and *d*). The

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specific activities of the corresponding six peaks from another similar run were as follows: *a*, 675 U./mg.; *b*, 722 U./mg.; *c*, 735 U./mg.; *d*, 760 U./mg.; *e*, 705 U./mg.; *f*, 680 U./mg. Within the limits of experimental error in the determinations of protein concentration and enzymatic activity, these specific activities are the same. The material eluted by the pH gradient is designated as Fraction I; that eluted by the NaCl gradient, Fraction II.

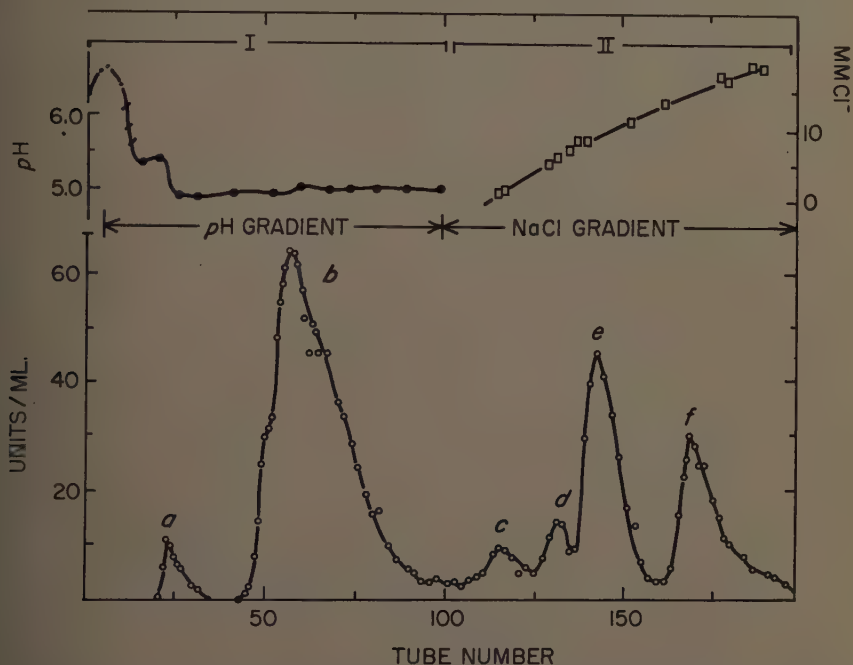


FIGURE 1. Anion exchange chromatography of yeast hexokinase on DEAE cellulose using gradient elution.³ Key: ○, hexokinase activity in units per ml; ●, pH; □, chloride ion concentration in effluent. Hexokinase, 21.5 mg. in 50 ml. of buffer (5×10^{-3} M succinate, pH 6.0) 6th crystals, 696 U./mg., placed on a column 9×60 mm. Tubes 0 to 4 contained the effluent collected during the addition of the enzyme. A pH gradient was established by placing pH 5.1 buffer (5×10^{-3} M succinate, 10^{-4} M versene) in mixing vessel (600 ml.) and running in buffer at pH 4.8, 5×10^{-3} M succinate. The pH of the effluent fell to 4.8 more abruptly than expected in this experiment. NaCl gradient 0 to 50 mM, pH 4.8 (5×10^{-3} M succinate). An air pressure bulb was used to increase the flow rate to approximately 0.2 ml. per min. Approximately 4 ml. per tube was collected.

Fraction II was never eluted unless a NaCl gradient was introduced, indicating that there is a distinct qualitative difference between these two fractions. On the other hand, the differences between the peaks within Fraction I or within Fraction II are rather subtle, and changes in the buffer concentration, column size, or rate of elution have a large influence on the elution pattern within the two major fractions.

In FIGURE 2*a*, a typical elution pattern of the crystalline yeast hexokinase from a DEAE-cellulose column, under the modified conditions used in most of the remaining experiments, is shown. In this experiment, the concentration of

the buffer was 10^{-3} *M* succinate (instead of 5×10^{-3} *M*), and the relative flow rate was twice as fast as that used in FIGURE 1. Under these conditions there is little separation within fractions.

Tests for artifacts. In order to eliminate the possibility of a column artifact as the cause of the occurrence of these two fractions, each fraction was separately chromatographed as shown in FIGURES 2*b* and *c*. It is clear from these data that each fraction is eluted at the expected position. It should be noted

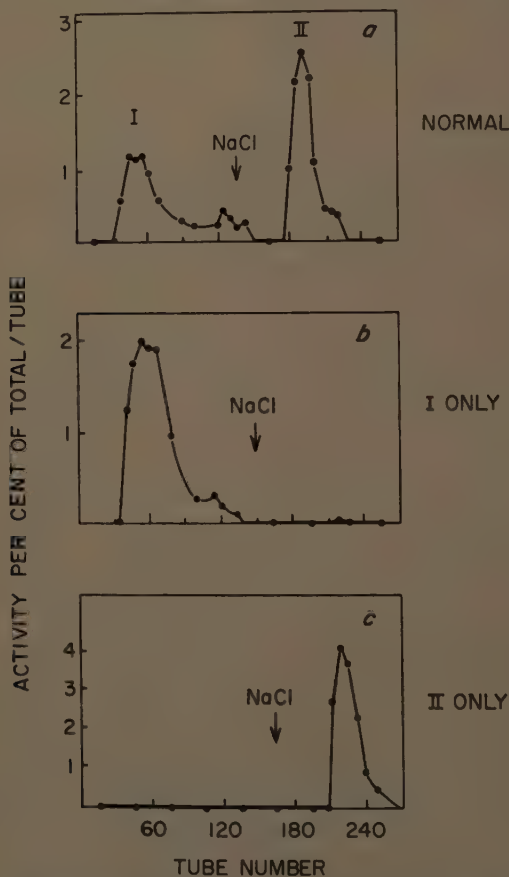


FIGURE 2. Rechromatography of Fractions I and II. In *a*, 1440 units of first crystalline suspension was dialyzed against $M/50$ Tris buffer (*pH* 8.0) followed by 10^{-3} *M* succinate buffer, *pH* 6.0. The solution was placed on a DEAE-cellulose column 8×90 mm. After loading with the hexokinase, the column was washed with succinate buffer (*pH* 6.0, 10^{-3} *M*). Effluent was collected from the time when gradient elution was started. A *pH* gradient was established by placing *pH* 5.1 buffer (10^{-3} *M* succinate) in mixing vessel (250 ml.) and running in buffer at *pH* 4.6 (10^{-3} *M* succinate). NaCl gradient, 0 to 50 mM, 10^{-3} *M* succinate *pH* 4.6. At the point of arrow the reservoir was switched to 50 mM NaCl, *pH* 4.6 (10^{-3} *M* succinate). Rate of flow was 2 ml./7.5 min. and every 2 ml. was collected. Assay of the enzymatic activity was done on 0.4 ml. of effluent taken from every fifth tube. The eluted Fractions I and II were kept in 30 per cent saturated ammonium sulfate solution after the *pH* was adjusted to 7.0. In *b*, 10,000 units of pooled Fraction I from several experiments was analyzed by the identical procedure. For hexokinase activity assay 0.1 ml. of the effluent was used. In *c*, 3,600 units of pooled Fraction II from several experiments was chromatographed.

also that the relative proportions of Fractions I and II are approximately 1:1 in the typical elution pattern (FIGURE 2a).

The possibility that these two fractions represent an artifact arising during extraction or purification was checked by the following experiments. As shown in FIGURE 3a, the crude extract used as the starting material for the usual preparation of the crystalline material gave a pattern upon chromatography similar to that found with the crystals. This indicates that the procedures used for preparation of the crystalline material from the crude extract do not alter the elution pattern of the enzyme. The possibility of an extraction artifact was checked by the use of a variety of extraction methods. FIGURES 3b, c, and d show the respective elution patterns of the crude extracts prepared as follows: (b) by applying a pressure (12 tons/sq. in.) to a sample of fresh baker's yeast in the cold (0 to 5° C.); (c) according to the classical Meyerhof method by toluene autolysis of fresh yeast, followed by extraction for 24 hours at 37° C.; (d) according to Lamanna and Mallette¹⁰ by stirring fresh yeast suspension with glass beads at high speed (in a Virtis Omnimixer) intermittently for a total of 3 min. in the cold. As shown in these elution patterns, there are always two fractions present in these crude extracts, regardless of the extraction method utilized. The ratio of Fraction I to II may vary as shown, but the variation is no greater than that observed in a series of preparations obtained by the standard method.

Single cell culture. In a separate experiment a yeast culture was grown from a single cell and the extract was obtained by the pressure method. The elution pattern of this extract clearly indicates the presence of the usual two Fractions I and II. Although there is still the remote possibility of mutation of the yeast during the course of growth, the result indicates that these two fractions do not represent the enzymes of two different strains of yeast cells.

*Electrophoresis of crystalline enzyme on starch gel.*³ Electrophoresis of 500 μ g. of the original form of hexokinase caused separation of 2 protein bands (amidol black stain), distinct but close together, about 3.8 cm. from the origin. A single broad yellow band that formed within 1 min. or 2 after overlaying with a gel containing a reaction mixture for colorimetric assay of hexokinase³ corresponded in position to the 2 closely spaced protein bands, probably indicating that activity was associated with both protein bands. A second, faint, narrow, yellow band formed more slowly 4.5 cm. from the origin. No protein band corresponding to this second band of enzymatic activity could be seen. All migrations were toward the anode. Although electrophoretic studies on the separated Fractions I and II have not been done, it is believed that the fast-moving protein corresponds to Fraction II, and the slow-moving one to Fraction I, since Fraction II appears to be the more acidic protein from its column behavior.

Comparative studies on Fractions I and II. Fractions I and II have both been crystallized under the same conditions as for the parent material, and they show the same crystal form.

As shown in TABLE 1, comparison of the Michaelis constants of these two fractions indicates no appreciable difference in these values, which are in good agreement with the values reported by Sols *et al.*¹¹

Urea and LiBr, which are believed to cause a decrease and increase, respec-

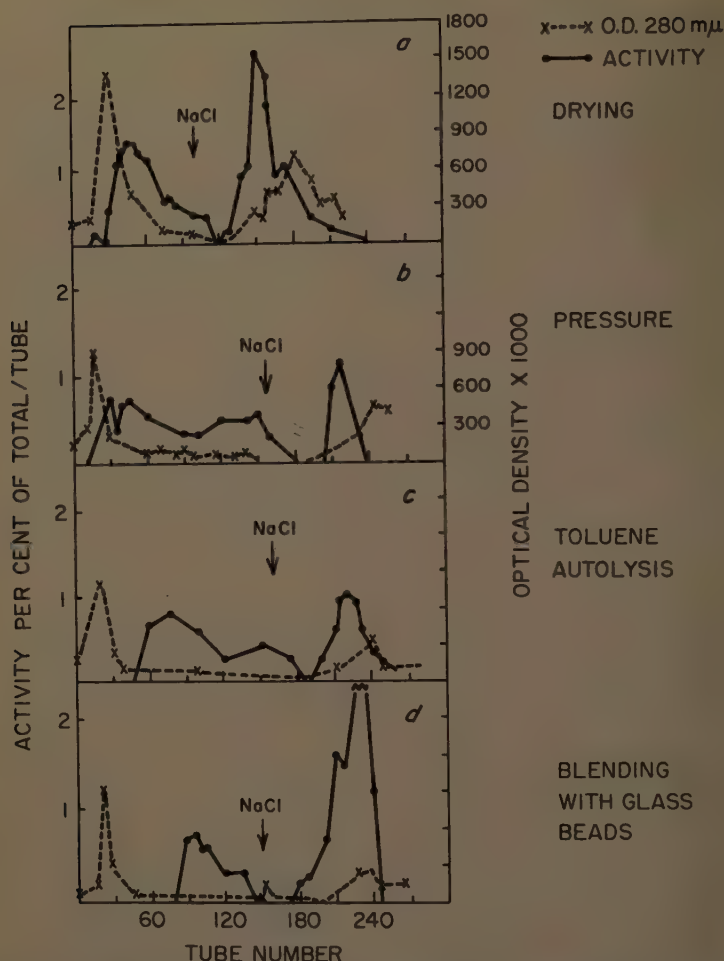


FIGURE 3. Elution pattern of crude yeast hexokinase extracted by four different methods. Key: ---X---X---, 280 $m\mu$ absorption, O.D. $\times 1000$; ———, hexokinase activity, per cent of total per tube.

(a) Fresh yeast was dried slowly at room temperature for 2 weeks. Then 333 g of the dried yeast was mixed with 1 l. of 0.2 M Na_2HPO_4 solution and the mixture was incubated for 3 hours at 37° C. with occasional stirring. The autolysate was centrifuged, first at 20,000 g for 60 min., and the supernatant fluid was further centrifuged at 100,000 g for 60 min. The final supernatant fluid was dialyzed against 10^{-3} M succinate buffer pH 6.0. Ten ml. of this solution containing 1100 U. of hexokinase activity was analyzed under the identical conditions described in the legend of FIGURE 2. The assay of the activity was done on 0.4 ml. of the effluent.

(b) Fresh yeast cake was packed up to the 40-ml. mark of a French press* that had been cooled to -60° C. To the packed yeast, 12 tons/inch² of pressure was applied at room temperature. During the entire operation, which lasted 20 to 30 min. the temperature of the extract was at most 15° C. To the extract, 20 ml. of cold distilled water was added, and the mixture was centrifuged and dialyzed. Five ml. of the solution containing 1665 U. was placed on a column and chromatographed as in a.

(c) One pound of yeast cake was mixed well with about 100 ml. of toluene by mortar and pestle. The excess toluene was squeezed out of the yeast and discarded. After incubation

* American Instrument Company, New York, N.Y.

tively, in the helical structure of proteins,¹² were tested on the activities of Fractions I and II in the hope that this might reveal a possible difference in secondary or tertiary structure between these fractions. As shown in FIGURE 4, there was no appreciable difference observed in the sensitivity of these two fractions toward these hydrogen bond-affecting agents.

Fractions I and II appear to be similar also with respect to accessibility of —SH groups. It was found that parahydroxymercuribenzoate inactivates both fractions at about the same rate when incubated at 30° C. Glucose protects both fractions from this type of inactivation.

The *pH*-activity curves of both fractions were studied and, as shown in FIGURE 5, there is no appreciable difference between them. The *pH* optimum for both Fractions I and II is about 7.2. The curve has a wide shoulder up to *pH* 9.0. The shape of the curve and the *pH* optimum were slightly different from that reported by Sols *et al.*¹¹

TABLE 1
COMPARISON OF MICHAELIS CONSTANTS* AND SPECIFIC ACTIVITIES
OF YEAST HEXOKINASE FORMS

Form	K_m for glucose	K_m for ATP	Specific activity
I	1.14×10^{-4}	1.25×10^{-4}	570 U./mg.
II	1×10^{-4}	9.4×10^{-5}	559 U./mg.

* For measurement of Michaelis constants, the cresol red, glycylglycine method⁴ was modified as follows. The final reaction mixture for determination of K_m for glucose was 1.66×10^{-3} *M* glycylglycine, 4.1×10^{-4} *M* ATP. The reaction mixture for determination of K_m for ATP was 1.66×10^{-3} *M* glycylglycine, and 2.9×10^{-3} *M* glucose. In the latter case, due to the varying buffering capacity at different ATP concentrations, each assay mixture had to be standardized by addition of a constant amount of acid.

It has been found previously³ that the crystalline hexokinase possessed a weak ATP-ase activity which consistently stayed with the hexokinase activity through six recrystallizations. Experiments were carried out to determine whether the fractions separated by the DEAE-cellulose column have ATP-ase activity to the same extent. Five times recrystallized hexokinase was placed on a DEAE-cellulose column as shown in FIGURE 6. Similar experimental conditions were used as in FIGURE 1. The effluent tubes containing the hexokinase were combined into six different fractions and each fraction was assayed for hexokinase and ATP-ase activity. The ATP-ase activity was measured by

at 37° C. for 2 hours, the yeast was mixed with 500 ml. of 0.1 *M* tris-(hydroxymethyl) amino-methane (Tris) buffer (*pH* 8.0), 1 per cent glucose solution. After incubation for 2 hours at 37° C., the mixture was centrifuged at 20,000 *g* and the supernatant was discarded. The residue was mixed with 500 ml. of the same Tris buffer solution containing glucose, and autolysis was allowed to proceed at 37° C. overnight. The autolysate was centrifuged and dialyzed, and 30 ml. of the solution containing 4800 U. was chromatographed as in *a*.

(*d*) Two hundred gm. of fresh Baker's yeast was mixed with 200 g of Ballotini glass beads and 40 ml. of 4 per cent glucose solution, 10^{-4} *M* sodium versenate solution (*pH* 7.8). The mixture was stirred at high speed with the container in ice (a Virtis Omnimixer was used). To prevent temperature rise, the stirring was performed only intermittently. Total stirring time was 3 min., and temperature was kept below 5° C. After the homogenization was completed, 200 ml. of the same glucose-versene solution was added. The mixture was centrifuged and dialyzed, and 20 ml. of the solution containing 1200 U. of the hexokinase was chromatographed as in *a*.

two independent methods, involving inorganic phosphate and ADP production, respectively. As shown in the inset, the ratio of these two activities is relatively constant throughout these six fractions. Additional evidence that the ATP-ase activity is an intrinsic characteristic of the hexokinase protein will be published elsewhere. It should be noted in FIGURE 6 that the absorption at $280\text{ m}\mu$ and the hexokinase activity follow each other closely, confirming the observation that the specific activities of all fractions are identical. The small amount of inactive protein which appeared between tubes 0 to 25 did not have either ATP-ase or hexokinase activity.

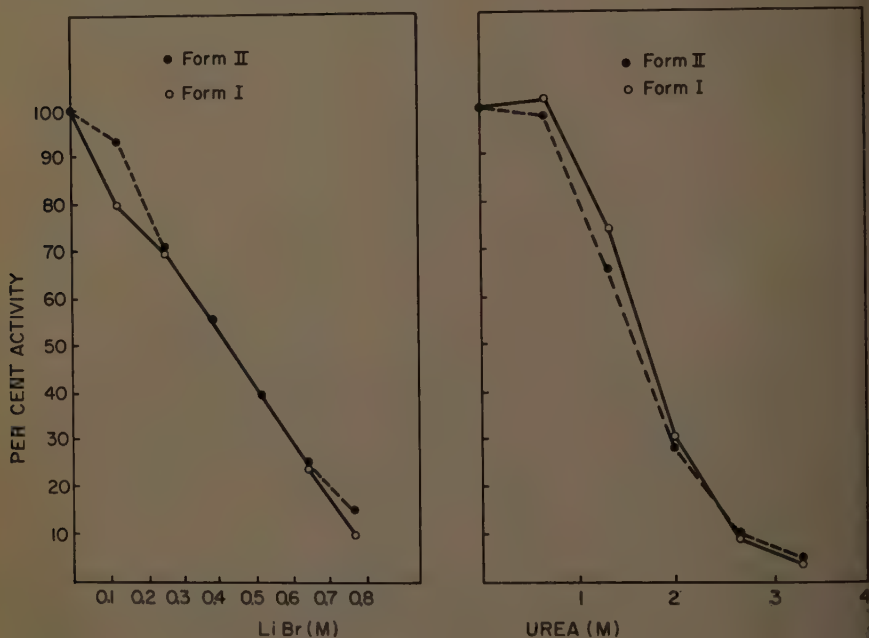


FIGURE 4. The effects of urea and LiBr on the hexokinase activities of Fractions I and II.

The reactions were started by the addition of about 4 U. of enzyme into the reaction mixture⁴ containing LiBr or urea as shown above. The buffering capacity of LiBr or urea at the pH used (8.5 to 9.0) is almost negligible.

Conversion of Fraction I to Fraction II. Attempts have been made to convert Fractions I and II into each other by the use of a variety of chemical treatments. Treatment of the original crystalline hexokinase with butanol, cysteine, glutathione, glucose, thiosulfate, sulfite, and cystine resulted in uniform failure to alter the chromatographic pattern. Finally, the use of proteolytic enzymes proved to be successful in converting Fraction I to Fraction II. Previously it has been reported¹³ that trypsin inactivates the yeast hexokinase and that glucose has a protective action against the inactivation. This observation has been confirmed and extended.³ The crystalline hexokinase was therefore treated with glucose and trypsin and fractionated by the usual DEAE-cellulose column chromatography. As shown in FIGURE 7a, all of the activity

of the enzyme applied on the column was now recovered as Fraction II and there was no Fraction I left after the treatment. A similar effect was observed by incubating 120 mg. of first crystals of hexokinase with 20 mg. of chymotrypsin in the presence of 0.1 *M* of glucose at 33° C. for 60 min.*

Unequivocal evidence that trypsin converts Fraction I to Fraction II is shown in FIGURE 8. In this experiment the isolated Fraction I was treated with trypsin and is shown to be converted to Fraction II. FIGURES 8*c* and *d* show respectively that the converted material is definitely distinct from the original Fraction I and chromatographically indistinguishable from natural Fraction II¹

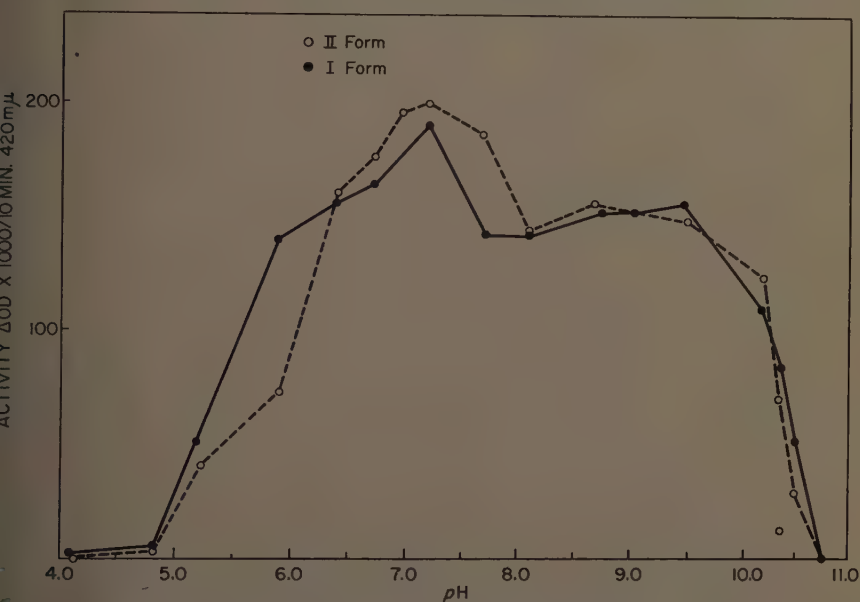


FIGURE 5. The pH activity curves of Fractions I and II. Reaction mixture for the assay of hexokinase activity contained 15 μ moles of ATP, 10 μ moles of $MgCl_2$, 6 μ moles of glucose, and 1 ml. of buffer solution in 3 ml. The buffer solution consisted of succinate, sodium phosphate, Tris, and glycine each 250 μ moles/ml. and the desired pH was obtained by addition of NaOH. The reaction was carried out at 30° C., and aliquots were taken every 6 min. for a period of 8 min. during which the reaction proceeded linearly. The free glucose content in the aliquots was estimated by measuring optical density at 420 $m\mu$ according to Hugget and Nixon.⁵

In order to test whether a similar proteolytic conversion takes place during the usual extraction as shown in the case of yeast phosphoglyceric acid mutase,¹⁶ crude extracts were prepared after various times of autolysis of the dried yeast

* In contrast to the report by other investigators,¹⁴ chymotrypsin was found to have the same inactivating effect as trypsin, although 10 times as much was needed; glucose has a protective effect against the chymotrypsin action, just as with trypsin. From studies carried out with soybean trypsin inhibitor it seems unlikely that the chymotrypsin effect is due to trypsin in the preparation. In view of the large amount of enzyme required to produce inactivation, the possibility still exists that the effect is due to some other proteolytic enzyme that may be present in the chymotrypsin preparation. This possibility, however, seems rather remote because two entirely different preparations gave similar results. (One from C. F. Boehringer and Son, GmbH, Mannheim, West Germany, and the other prepared in Neurath's laboratory at the University of Washington, Seattle, Wash. and kindly supplied by Jan Van Vlyss of the Department of Biochemistry, Vanderbilt University School of Medicine.)

in phosphate solution at 37° C. and the hexokinase elution pattern of these crude extracts was compared. As shown in FIGURE 9, there is no appreciable difference between a 30-min. autolysate and a 3-hour autolysate and, even after a 24-hour autolysis, an appreciable amount of Fraction I was present (see also

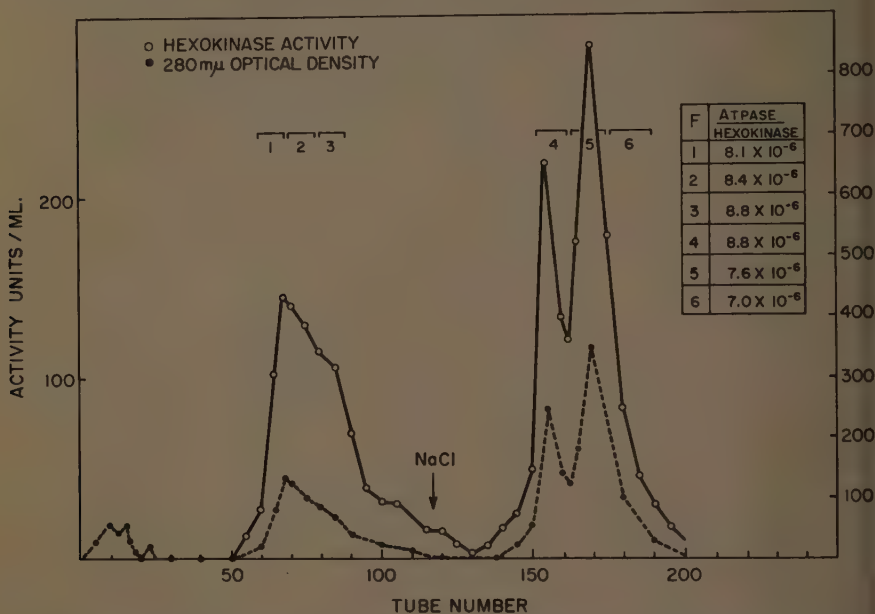


FIGURE 6. Elution pattern of 5 times recrystallized yeast hexokinase and comparison of ratio ATP-ase/hexokinase of each fraction. The hexokinase was dissolved in $5 \times 10^{-3} M$ succinate, pH 6.0, dialyzed against the same buffer, and the solution containing about 290 mg. of protein was placed on a 2.2×6 cm. column of DEAE cellulose (succinate form). Effluent was collected from the time of loading the column with the enzyme. A pH gradient was established by placing pH 5.1 buffer $5 \times 10^{-3} M$ succinate, versene $10^{-4} M$ in mixing vessel (2.2 l.) and running in buffer at pH 4.6, $5 \times 10^{-3} M$ succinate. At the 64th tube the reservoir was switched to pH 4.5, $5 \times 10^{-3} M$ succinate, and at the 117th tube to pH 4.5, $5 \times 10^{-3} M$ succinate, 100 mM NaCl. At the 151st tube, the reservoir was further changed to pH 4.5, $5 \times 10^{-3} M$ succinate, 100 mM NaCl. The rate of flow was 15 ml./7.5 min. and every 15 ml. was collected. Tubes 60 to 69, 70 to 79, 80 to 89, 152 to 162, 163 to 175, and 176 to 190 were combined into fractions 1 to 6 respectively. Each fraction was concentrated by dialysis against 15 to 30 per cent polyvinylpyrrolidone, $10^{-2} M$ cysteine at pH 7.0 for 30 hours. About 30 per cent of the enzymatic activity was lost by the concentration process. For ATP-ase assay, reaction mixture contained 1 μ mole of ATP, 20 μ moles of $MgCl_2$, 100 μ moles of Tris buffer (pH 8.0) and about 0.5 mg. of enzyme per 1 ml. The reaction was carried out at 30° C. for 1 hour, and aliquots were taken every 20 min. for inorganic phosphate assay. Hexokinase activity was measured by the usual dye method⁴ after proper dilution of the enzyme. The ratio of these two activities is shown in the inset. Key: ○—○, hexokinase activity unit/ml. effluent; ●—●, optical density $\times 1000$ at 280 $m\mu$. Right hand ordinate.

FIGURE 3c). However, there is some suggestion in FIGURE 9 that a predominant amount of Fraction II is present in the 24-hour autolysate, indicating that conversion, though slow, may take place. This predominance of Fraction II was more apparent in a separate experiment where a crystalline preparation was made from the 24-hour autolysate. Although proteolytic conversion may occur upon prolonged extraction, the rate is so small that the presence of Frac-

ions I and II in the 30-minute extract does not seem to be attributable to a change from I to II by proteolytic action during extraction.

Discussion

In the study of multiple forms of enzymes, one must consider the possibility of at least three kinds of artifacts. These are: column artifacts, extraction

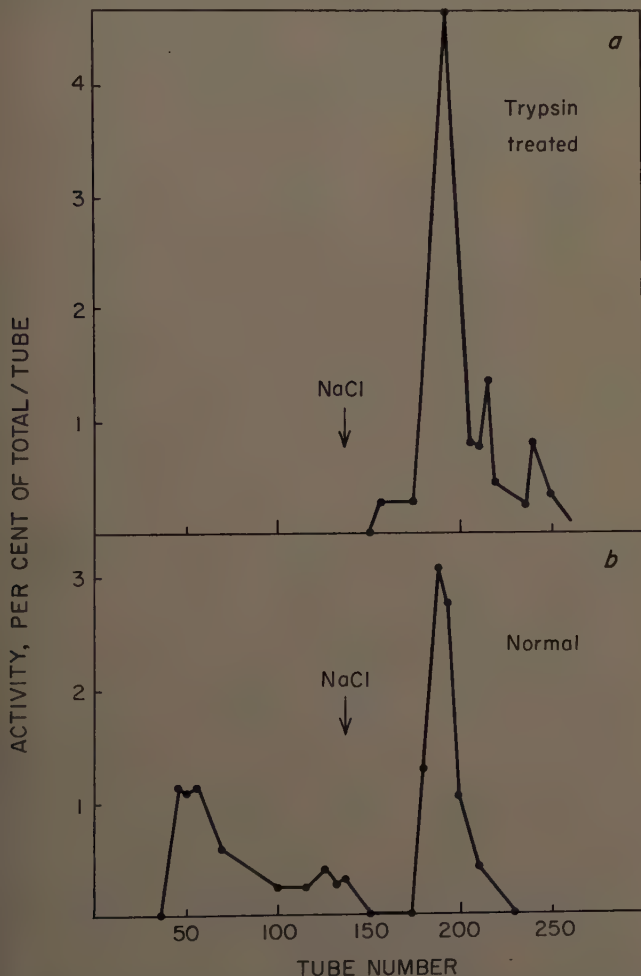


FIGURE 7. Effect of trypsin on elution pattern of the hexokinase. (a) Crystalline hexokinase (about 40 mg.) was mixed with 8 mg. of twice recrystallized trypsin* in 0.1 *M* glucose, 5×10^{-4} *M* versene, 1/50 *M* Tris buffer (pH 8.0) and incubated for 90 min. There was no loss of enzymatic activity during the trypsin treatment. After the incubation the mixture was dialyzed against 10^{-3} *M* succinate pH 6.0, 10^{-2} *M* glucose solution for 5 hours and placed on a DEAE-cellulose column as described in FIGURE 2. The elution was carried out as usual except that the buffer contained 10^{-2} *M* glucose.

(b) Normal elution pattern before treatment with trypsin. Experimental conditions are identical to those of FIGURE 2a.

* From Worthington Biochemical Sales Co., New York, N.Y. In a separate experiment, identical results were obtained with much less trypsin (2 per cent of the hexokinase).

artifacts, and purification artifacts. These possibilities must be carefully examined before the decision is made as to the existence of multiple forms of an enzyme in organisms. Studies with D-glyceraldehyde-3-phosphate dehydrogenase¹⁶ and ribonuclease¹⁷ respectively, show clearly that column and purification artifacts are responsible in some cases for the presence of multiple forms.

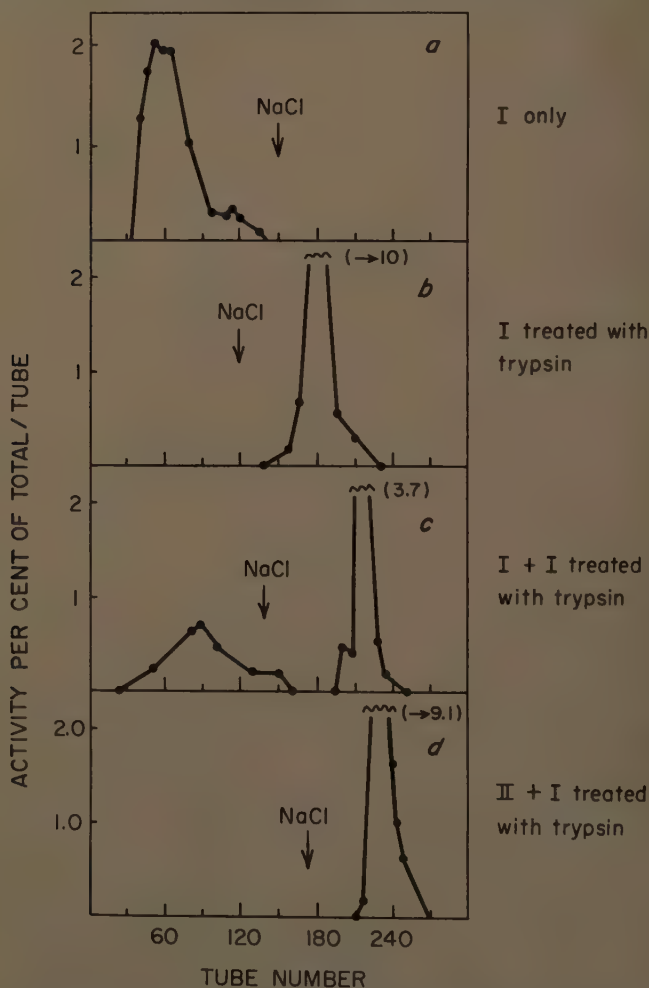


FIGURE 8. Evidence for conversion of Fraction I to Fraction II by trypsin treatment.

(a) The elution pattern of isolated Fraction I. Ten thousand U. of pooled Fraction I was chromatographed by the procedure described in FIGURE 2.

(b) About 30 mg. of pooled Fraction I was mixed with 20 mg. of trypsin in 0.1 M glucose, 1/50 M Tris buffer (pH 8.0) and incubated for 90 min. at 33° C. The mixture was dialyzed and analyzed chromatographically as described in FIGURE 7a.

(c) One thousand five hundred U. of the converted fraction (the material obtained in b) and 1,680 U. of original Fraction I (the same material as used in a) were mixed and chromatographed as in 8a.

(d) One thousand six hundred U. of Fraction II was mixed with 1300 U. of the converted fraction (the material obtained in b) and analyzed chromatographically under the same conditions as above.

In the present case the demonstration of a single peak on rechromatography rules out the possibility of a column artifact. The existence of two distinct fractions in extracts prepared by four independent and relatively mild extraction procedures makes it very unlikely that an extraction artifact is responsible for the multiple forms. The comparison of the elution pattern of crude extract and that of the crystalline material makes it clear that the purification steps do not produce these two major fractions. Although there is still a

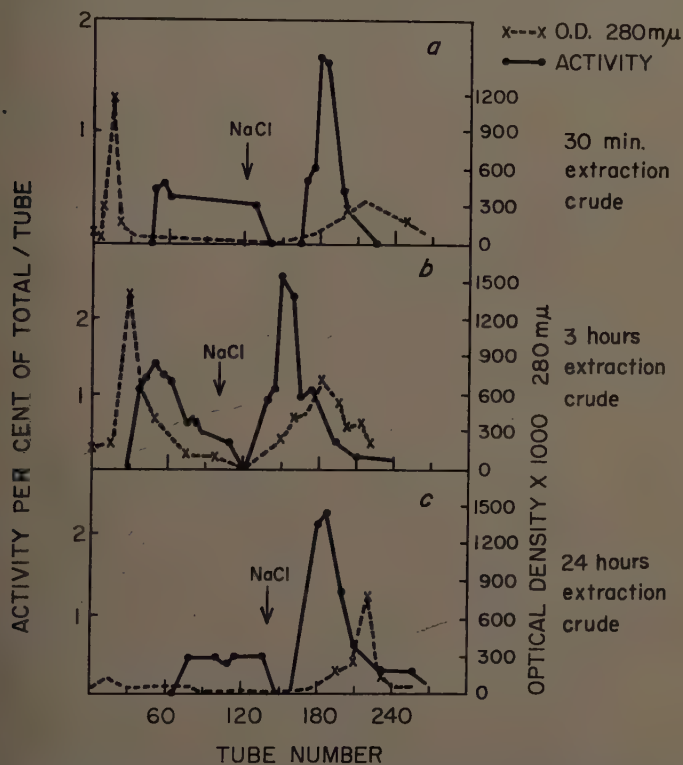


FIGURE 9. Relationship between period of autolysis and the elution pattern of crude yeast hexokinase. Extraction of the crude hexokinase was carried out as described in FIGURE 3a with the exception of period of autolysis. The chromatography of the extract was done as in FIGURE 3a. Key: \bullet — \bullet , hexokinase activity, per cent of total/tube; \times — \times — \times , 280 mμ sorption. Nine hundred and thirty-six U., 1100 U., and 930 U. of the enzyme were analyzed by the DEAE-cellulose column in *a*, *b*, and *c* respectively.

remote possibility that Fraction II is the product of yeast proteolytic action on Fraction I during extraction, the slowness of conversion during the extraction makes this possibility rather unlikely. If a converting enzyme does occur in yeast, it would be worthy of study, since this could well provide the physiological mechanism by which these two fractions are produced in the organism. Assuming the presence of these two fractions in organisms, the physiological significance of these multiple forms of hexokinase remains totally obscure. In view of the findings by Stadtman *et al.*¹⁸ it is still possible that the presence of these two hexokinases is important in metabolic regulation of alternative path-

ways of metabolism in the yeast cell. A number of metabolic intermediates and products should be tested for their differential inhibitory effect on Fractions I and II.

All of the present attempts to find catalytic differences between Fractions I and II have failed. The pH optima, sensitivity toward agents affecting hydrogen bonding or, sulfhydryl groups, Michaelis constants, and specific activities of the Fractions I and II were identical within experimental error.

The conversion of Fraction I to the more acidic Fraction II by trypsin or chymotrypsin in the presence of glucose indicates that the conversion probably involves the removal of a peptide moiety rich in basic amino acids. Amino acid analysis of the two fractions, to check this possibility, is in progress. A number of cases have been reported where fragments of enzyme produced by proteolytic digestion still maintain part of their enzymatic activity (for examples, see Hill and Smith¹⁹). The fact that chymotrypsin and trypsin cause similar conversion suggests, of course, that the products are not truly identical. In view of the heterogeneity of Fraction II, as shown in FIGURE 1, there is a possibility that the chymotrypsin-treated material and trypsin-treated material will prove to be separable chromatographically, though they are both eluted by the NaCl gradient. In either case, it is clear that the peptide that is split off is not required for the enzymatic activity. The nature of this conversion is currently under investigation.

Summary

Yeast hexokinase has been separated into several fractions by DEAE-cellulose column chromatography. The major two fractions (I and II) have been obtained by pH gradient and NaCl gradient elution respectively. These two fractions are identical with respect to a number of characteristics such as K_M , pH optima, sensitivity to inhibitors, and specific activity of hexokinase as well as ATP-ase. Fraction I can be converted to Fraction II by trypsin or chymotrypsin treatment in the presence of glucose.

Acknowledgment

We express our appreciation to Howard C. Mitchell and Clark Simmons, who prepared the hexokinase used in this study through the first crystallization step.

References

1. PALEUS, S. & J. B. NEILANDS. 1950. *Acta Chem. Scand.* **4**: 1024.
2. KREBS, E. G. 1953. *J. Biol. Chem.* **200**: 471.
3. TRAYSER, K. A. & S. P. COLOWICK. *Arch. Biochem. Biophys.* In press.
4. DARROW, R. A. & S. P. COLOWICK. 1961. *In Methods in Enzymology*, Volume V. S. P. Colowick & N. O. Kaplan (Editors). In press. Academic Press. New York, New York.
5. HUGGET, A. STG. & D. A. NIXON. 1957. *Biochem. J.* **66**: 12P.
6. CHEN, P. S., JR., T. Y. TORIBATA & H. WARNER. 1956. *Analytical Chem.* **28**: 1956.
7. CRANE, R. K. & F. LIPMANN. 1953. *J. Biol. Chem.* **201**: 235.
8. KORNBERG, A. & W. PRICER, JR. 1951. *J. Biol. Chem.* **193**: 481.
9. BÜCHER, T. & G. PFLEIDERER. 1955. *In Methods in Enzymology*, Volume I. S. P. Colowick & N. O. Kaplan (Editors): 435. Academic Press. New York, New York.
10. LAMANNA, C. & M. F. MALLETT. 1954. *J. Bacteriol.* **67**: 503.
11. SOLS, A., G. DE LA FUENTE, C. VILLAR-PALAST & C. ASENSIO. 1958. *Biochim. Biophys. Acta* **30**: 92.

2. HARRINGTON, W. F. & J. A. SCHELLMAN. 1956. Compt. Rend. Trav. Lab. Carlsberg Ser. Chim. **30**: 167.
3. BERGER, L., M. W. SLEIN, S. P. COLOWICK & C. F. CORI. 1946. J. Gen. Physiol. **29**: 397.
4. KUNITZ, M. & M. R. McDONALD. 1946. J. Gen. Physiol. **29**: 393.
5. CHIBA, H., E. SUGIMOTO & M. KITO. 1960. Bull. Agr. Chem. Soc. Japan. **24**: 556.
6. BOROSS, L., T. KELETI & M. TELEGD. 1960. Acta Physiol. Acad. Sci. Hung. **2**: 15.
7. SHAPIRA, R. & S. PARKER. 1960. Biochemical Biophysical Research Communications. **3**: 200.
8. STADTMAN, E. R., G. N. COHEN, G. LEBRAS & H. DE ROBICHON-SZULMAJSTER. 1961. This conference.
9. HILL, R. L. & E. L. SMITH. 1958. J. Biol. Chem. **231**: 117.

THE ISOLATION AND KINETICS OF TWO FORMS OF FUMARASE FROM TORULA YEAST*

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Since the mechanism of action of pork-heart fumarase has been investigated in considerable detail,¹ it would seem valuable to compare the properties of this enzyme with those of a fumarase isolated from a very dissimilar source. In the course of the isolation of the enzyme from torula yeast, it became apparent that the enzyme occurred in two active forms, and a further study of the two forms was undertaken.

Experimental

Materials. The yeast used was *Candida utilis* (formerly known as *Torulopsis utilis*) produced commercially on spruce wood sugars† and air-dried by the manufacturers at 37° C. L-Malic acid was A-grade,‡ and was not purified further for assays performed during the course of the enzyme purification. For kinetic experiments it was recrystallized from ethyl acetate and petroleum ether.² Fumaric acid was recrystallized from water and tris(hydroxymethyl)aminomethane (abbreviated as Tris) from 95 per cent ethanol. All other reagents were reagent-grade. Pork-heart fumarase was prepared by a salt fractionating procedure.³ Ethanolyzed cellulose was purchased from the Grycksbo Pappersbruk,§ and type-40 diethylaminoethyl cellulose (DEAE cellulose) from the Brown Company.||

Methods. The enzymes were assayed as described in Frieden *et al.*³ by following the rate of appearance of fumarate by means of the increase in ultra-violet absorbency as measured with a Cary Model No. 14 recording spectrophotometer equipped with a 0 to 0.1 absorbency slide wire. The concentrations of the yeast enzymes were expressed as if they have the same turnover number per gram as pork-heart fumarase. Since the torula fumarases have not been crystallized and since the data on turnover number that are available indicate that this means of expression is not too inaccurate, it has been retained. Protein concentration was measured by determining the absorbency at 280 μ in a 1 cm. cuvette.

Column electrophoresis was performed according to the technique of Porath⁴ on a 3 \times 150 cm. column, packed with ethanolyzed cellulose and cooled by water circulating from a 5° C. bath. The power supply was designed and built by the Electrical Engineering Department of the University of Wisconsin, according to the specifications of William McShan of the Zoology Department.

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† By the Lake States Yeast and Chemical Division, St. Regis Paper Company, Rhineland, Wis.

‡ From the California Corporation for Biochemical Research, Los Angeles, Calif.

§ Grycksbo, Sweden.

|| Berlin, N. H.

For DEAE cellulose chromatography, the resin was packed in a small condenser of one-half inch diameter. The ultracentrifuge experiments were run in a Spinco model-E ultracentrifuge using a partition cell with a metal plate.

All kinetic determinations were made at 25° C., using 0.01 *M* Tris acetate buffer. Ten-cm. cuvettes were used to insure that there would be sufficient change in absorbency during the initial portion of the reaction. Concentrations of substrate were chosen to span the range of one fifth to five times the Michaelis constants in so far as possible, since this range of concentrations usually yields initial velocities that obey the Michaelis-Menten equation. It was found that consistent maximum velocities were obtained when a series of solutions over a range of *pH* values were made up at an arbitrary relatively high substrate concentration, and initial velocities were determined, and then maximum velocities were calculated from the Michaelis-Menten equation, using the values for Michaelis constants read from a smooth curve of previously determined Michaelis constants versus *pH*. All maximum velocities were normalized to the same enzyme concentration by dividing them by the initial velocity of the standard that consisted of 100 μM malate in 0.01 *M* Tris acetate buffer at *pH* 7.4. Since the absolute enzyme concentrations were unknown for the two torula enzymes, no effort was made to express the results in terms of turnover per enzyme molecule. Since the enzymes exhibited a fairly high loss of activity initially, followed by somewhat greater stability, they were stored in solution in 0.01 *M* Tris acetate buffer, *pH* 7.4, in plastic tubes in an ice bath for at least one hour before the start of the experiment. In order to insure the best possible intercomparison of the kinetic parameters, virtually all the determinations were made upon the three enzymes concurrently, using the enzymes alternately with standards to permit correction for loss of enzyme activity during the duration of an experiment. Michaelis constants were calculated by means of a least-squares fit of a plot⁵ of v/s versus v , where v is the initial velocity and s the substrate concentration. Although the use of a least-squares calculation resulted in some degree of arbitrary weighting, the treatment of the data was consistent. The data were less reliable at low *pH* values where the substrate concentrations were lower and the enzymes less stable.

Preparation of the Enzymes

Extraction and salt fractionation. Stirring a suspension of the dried yeast in dilute buffer for a few hours at room temperature proved to be a simple and effective way of extracting the enzymes. Fifty gm. of dried torula yeast were washed with 150 ml. of water for 30 min. and then centrifuged and resuspended in 500 ml. of 0.02 *M* sodium phosphate buffer, *pH* 7.3. After the suspension had been stirred for 5 to 6 hours, 5 gm. of manganese sulfate were added to precipitate nucleic acids, and the suspension was centrifuged. All ensuing steps were at 5° C. The clear orange supernatant was dialyzed overnight against sufficient saturated ammonium sulfate to achieve a final concentration of 75 per cent of saturation. The precipitated enzyme was then suspended in 10 ml. of 20 per cent saturated ammonium sulfate (all dilutions of ammonium sulfate were made with 0.01 *M* sodium phosphate buffer, *pH* 7.3) and dialyzed for several hours against an appropriate ammonium sulfate concentration to a final value of 45 per cent of saturation. The precipitate was discarded, and

the supernatant enzyme was reprecipitated by dialysis to 75 per cent saturation of ammonium sulfate. At the conclusion of column fractionations, the pooled enzymes were reprecipitated by dialysis to 85 per cent saturation of ammonium sulfate.

Column electrophoresis. The column was equilibrated with 0.05μ sodium phosphate buffer, pH 7.6, by washing with 1 or 2 bed volumes of the buffer. The crude enzyme, which had been dissolved in a minimal volume of a dilute neutral phosphate buffer, about $0.002 M$ and dialyzed overnight against several changes of the same buffer, was carefully applied to the top of the column and washed in with 10 ml. of distilled water followed by the 0.05μ sodium phos-

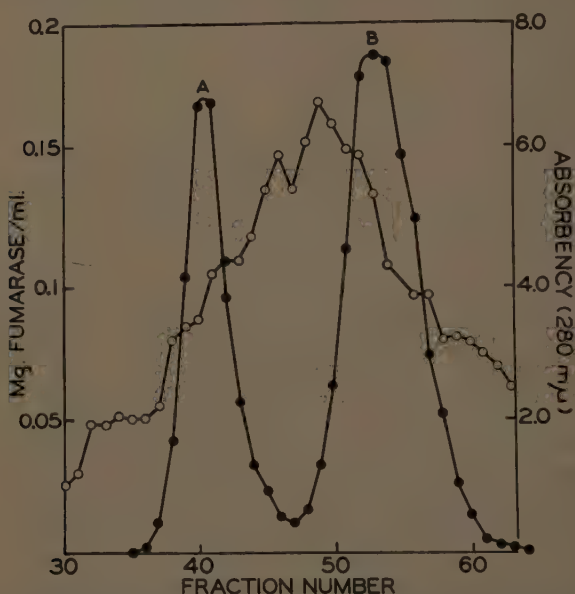


FIGURE 1. Column electrophoresis of crude torula fumarase. The enzyme migrated toward the positive pole. The left ordinate designates the enzyme concentration (closed circles), and the right ordinate the protein concentration (open circles). The buffer was 0.05μ sodium phosphate, pH 7.6, and electrophoresis was conducted for 45 hours at 50 mAmp. current. Ten-ml. fractions were collected at a flow rate of 1 ml. per min.

phate buffer. When 50 ml. of solution had flowed from the column, flow was shut off, the entire top of the column was filled with buffer, the electrodes were connected so that the positive pole was at the bottom of the column, and electrophoresis was begun and usually continued for 60 hours at 50 mAmp. current with interruptions every 12 hours to permit mixing the contents of the two electrode vessels. The enzymes were eluted in 10-ml. fractions at a flow rate of 1 ml. per min. The results of 4 column-electrophoresis experiments may be seen in FIGURES 1 through 4. In FIGURE 1, 2 gm. of crude torula fumarase were applied to the column and allowed to migrate for 45 hours. The enzyme activity broke into 2 well-separated peaks with the protein distributed over the entire column, its maximum between the 2 enzyme peaks. Each enzyme, fraction A, the more rapidly migrating, and fraction B, was pooled and rerun on

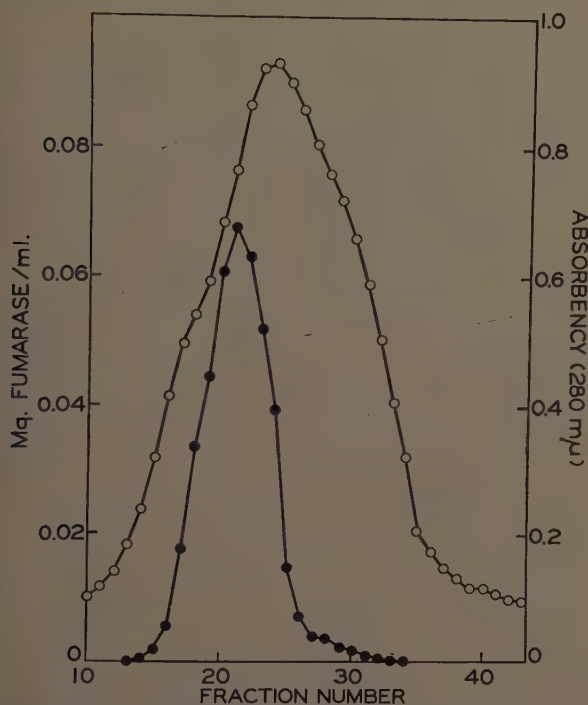


FIGURE 2. Column electrophoresis of fraction A. The same conditions and symbols as FIGURE 1, except that electrophoresis was continued for 60 hours.

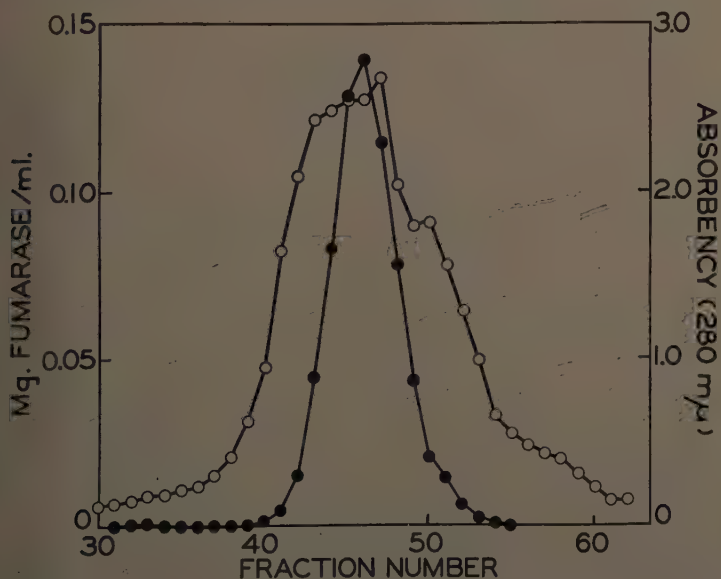


FIGURE 3. Column electrophoresis of fraction B. The same conditions and symbols as FIGURE 2.

the column. Neither fraction A (FIGURE 2) nor fraction B (FIGURE 3) showed any sign of further fractionation after 60 hours of electrophoresis, although there is visible a trace of fraction A to the left of the main enzyme peak in FIGURE 3. When the 2 fumarases were recombined and once again subjected to electrophoresis, they separated into two peaks of the same mobilities as they exhibited initially (FIGURE 4). Thus the existence of the 2 fractions cannot be attributed to an aggregation or dissociation on the column.

Cellulose Ion-Exchange Chromatography

DEAE cellulose chromatography could be employed effectively either prior to or after column electrophoresis. The precipitated enzyme was dissolved in a minimal volume of 0.01 *M* sodium phosphate buffer, pH 7.5, and dialyzed against several changes of the same buffer. The column was packed with

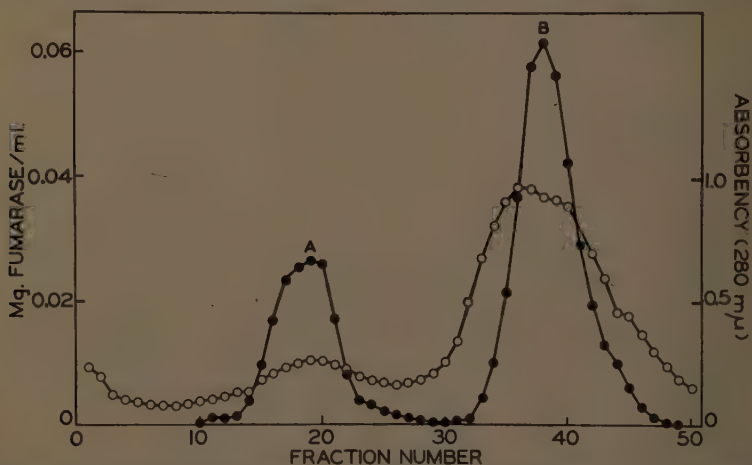


FIGURE 4. Column electrophoresis of recombined fractions A and B. The same conditions as in FIGURE 2.

about 5 gm. of DEAE cellulose, which had been washed with water and allowed to settle several times to eliminate the finest particles, and was equilibrated with the same buffer. After the application of the enzyme to the column and a washing-in with about 20 ml. of buffer, elution was started with a linear gradient of sodium chloride. Ten-ml. fractions at a flow rate of 1 ml. per min. were collected. A typical result with crude fumarase is shown in FIGURE 5. As in column electrophoresis, two enzyme peaks are apparent, but fraction B was the first to emerge from the column, as one might expect from its less negative charge. Repeated DEAE cellulose chromatography of fraction B revealed indications of inhomogeneity, as shown in FIGURE 6. Several ill-defined peaks are visible, but no further resolution was obtained. This sort of behavior may be due to the pooling of fraction Bs from several crude preparations after column electrophoresis, but similar treatment of fraction A caused no appearance of inhomogeneity. It is possible that the inhomogeneity of fraction B might be due to adsorption of contaminants that might be different in enzymes of slightly dissimilar histories.

The best preparation of fraction B had a specific activity of 0.4, expressed as equivalent milligrams of pork-heart fumarase per milliliter per unit of absorbency of the solution at 280 $m\mu$ with a 1 cm. path length. If one assumes

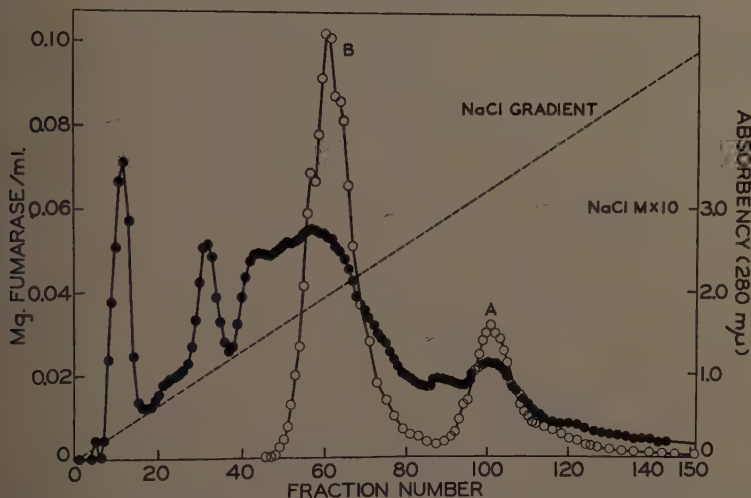


FIGURE 5. DEAE cellulose chromatography of crude torula fumarase. The left ordinate designates the enzyme concentration (*open circles*), and the right ordinate the protein concentration (*closed circles*) and sodium chloride concentration (*dashed line*). Ten-ml. fractions were collected at a flow rate of 1 ml. per min.

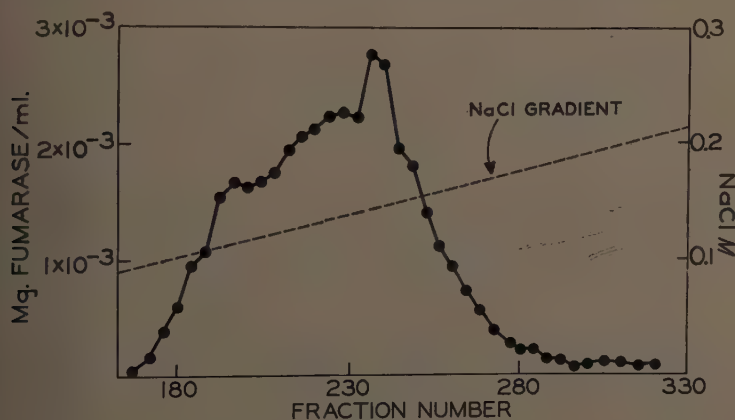


FIGURE 6. DEAE cellulose chromatography of fraction B. About 5 to 10 mg. of protein were applied to the column. The right ordinate designates enzyme concentration.

that a solution containing 1 mg. of protein per milliliter has an absorbency of 1 and that the turnover number of torula fumarase is equal to that of pork-heart fumarase, this preparation would be about 40 per cent pure. From the appearance of the chromatographic results of the experiment by means of which this sample was prepared, the material was obviously less than 100 per cent pure. The most highly purified sample of fraction A had a specific activity

of 0.2. However, for kinetic experiments, enzymes with specific activities of 0.01 to 0.05 were used.

Sedimentation Studies

Approximate values for the sedimentation coefficients of the two impure fractions of torula fumarase were obtained by means of centrifugation in a ultracentrifuge cell, followed by determination of the activity distribution in the ultracentrifuge cell.

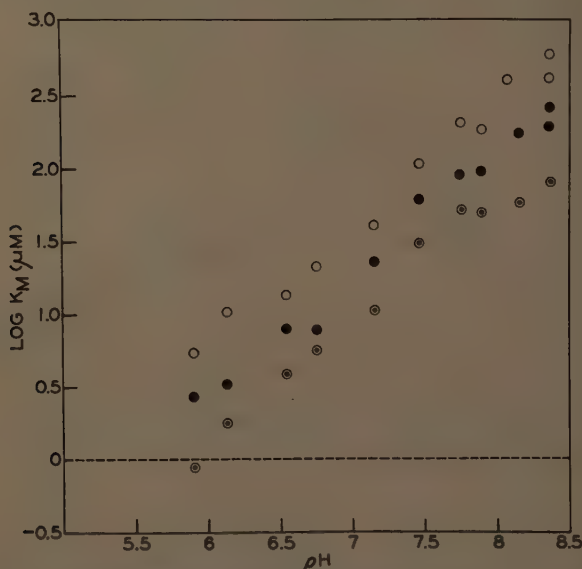


FIGURE 7. A plot of the logarithm of K_M versus pH for fraction A (open circles), fraction B (closed circles), and pork-heart fumarase (dotted circles).

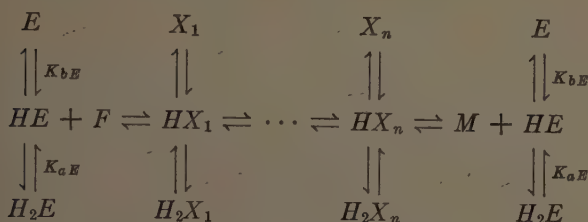
tracentrifuge cell. The sedimentation coefficients were calculated with the aid of the following equation⁶

$$s = \frac{1}{\omega^2 t} \ln \left[\frac{x_0^2}{x_p^2} + \frac{c_t}{c_0} \left(1 - \frac{x_0^2}{x_p^2} \right) \right]$$

where s is the sedimentation coefficient in reciprocal seconds, ω is the angular velocity, x_0 the distance in centimeters from the center of rotation to the partition, t the time in seconds at full speed, c_t the concentration of active material in the top compartment of the cell at the conclusion of centrifugation, and c_0 the concentration of activity in the original solution. About 5 or 10 mg. of each enzyme were sedimented at 13° C. in sodium phosphate buffer, pH 7.3, 0.05 M . for one-half hour at 59,700 rpm. The sedimentation coefficients for fractions A and B respectively were 9.5 and 9.7 Svedberg units, which are identical within experimental error.

Results

Pork-heart-fumarase data have been shown to conform reasonably well to the following mechanism⁷



where the enzymatic site is considered to be a dibasic acid with the singly protonated form being the active one. F and M are totally dissociated fumarate

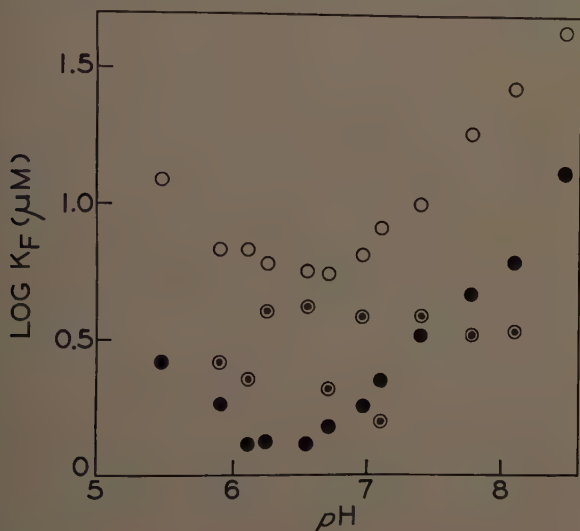


FIGURE 8. A plot of the logarithm of K_F versus pH for the three enzymes with the same symbols as in FIGURE 7.

and malate. The ionization constants of the free enzymatic site, K_{aE} and K_{bE} , and apparent ionization constants, K'_{aEF} , K'_{bEF} , K'_{aEM} and K'_{bEM} , for the enzyme-substrate complexes have been determined from plots of V versus pH and V/K versus pH with the aid of the following equations:

$$\begin{aligned}
 V_F &= \frac{V_F'(E)_0}{1 + (H^+)/K'_{aEF} + K'_{bEF}/(H^+)} \\
 V_M &= \frac{V_M'(E)_0}{1 + (H^+)/K'_{aEM} + K'_{bEM}/(H^+)} \\
 K_F &= K_F' \frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K'_{aEF} + K'_{bEF}/(H^+)} \\
 K_M &= K_M' \frac{1 + (H^+)/K_{aE} + K_{bE}/(H^+)}{1 + (H^+)/K'_{aEM} + K'_{bEM}/(H^+)}
 \end{aligned}$$

where V_F and V_M are the experimentally determined maximum velocities for fumarate and malate; K_F and K_M the Michaelis constants for fumarate and

malate; and V_F' , V_M' , K_F' , K_M' the corresponding pH independent parameters. We have studied the kinetics of the torula fumarases and pork-heart fumarase in order to intercompare their Michaelis constants, maximum velocities, and ionization constants.

A plot of the logarithm of K_M versus pH for the three enzymes is shown in FIGURE 7. Throughout the pH range there is a significant difference in the

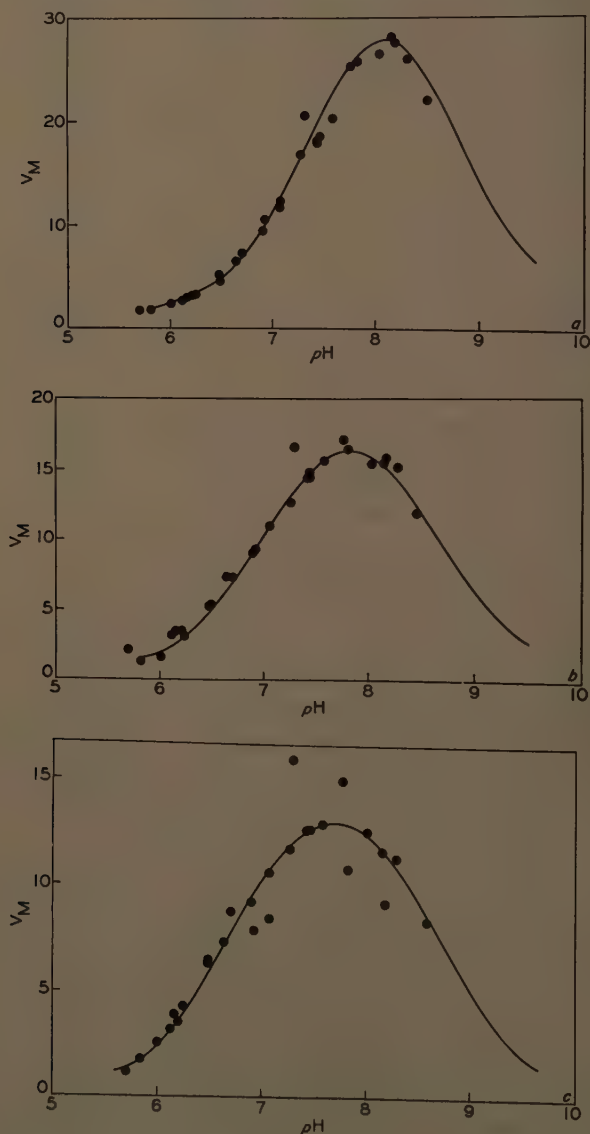


FIGURE 9. Plots of $V_M (\times 10)$ versus pH for fraction A (a), fraction B (b), and pork-heart fumarase (c). Values have been corrected for enzyme concentration by division by the initial velocity of the standard.

values for the three enzymes. In general the values for fraction A are twice those for fraction B, and four times those for the pork enzyme. In FIGURE 8 here is a similar plot of K_F . In this case the results for fraction A are considerably higher than those for fraction B, but the pork-heart values are scattered somewhere between. The plots of V_M versus pH are in FIGURES 9a to c for fraction A, fraction B, and pork-heart fumarase respectively. In all cases the plots are bell-shaped and can be fit by use of the above equation, as is shown by the solid lines. The pH optima for the three enzymes are 8.1, 7.8, and 7.7 for fraction A, fraction B, and pork-heart fumarase respectively. Although the differences are small, they are significant. The plots of V_F versus pH are shown in FIGURE 10. In this case only one solid line is plotted, that

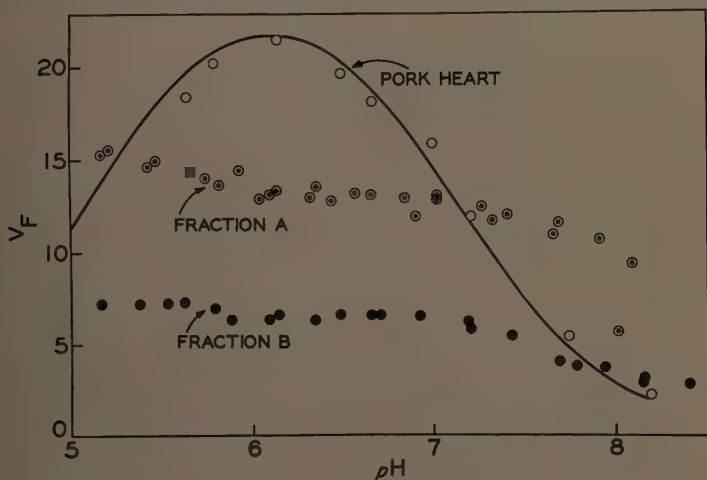


FIGURE 10. Plots of $V_F (\times 10)$ versus pH for the three enzymes.

for pork-heart fumarase, because the data for fractions A and B do not fit a bell-shaped curve and cannot be described by the usual equation. For neither enzyme does V_F vary much with pH ; there appears merely to be a gradual decrease with increasing pH until pH 8, when there occurs a more rapid decline.

In FIGURES 11a to c may be seen the plots of

$$V_M/K_M \left(1 + \frac{(H^+)}{K_{HM}} \right)$$

and

$$\frac{V_F}{4.4K_F} \left(1 + \frac{(H^+)}{K_{HF}} \right)$$

for the three enzymes in the usual order. According to the Haldane relation the fumarate and malate plots should be superimposable. This requirement is met for fraction B and pork-heart fumarase, but there is a marked deviation in the case of fraction A. The deviation may possibly be due to nonrandom experimental error.

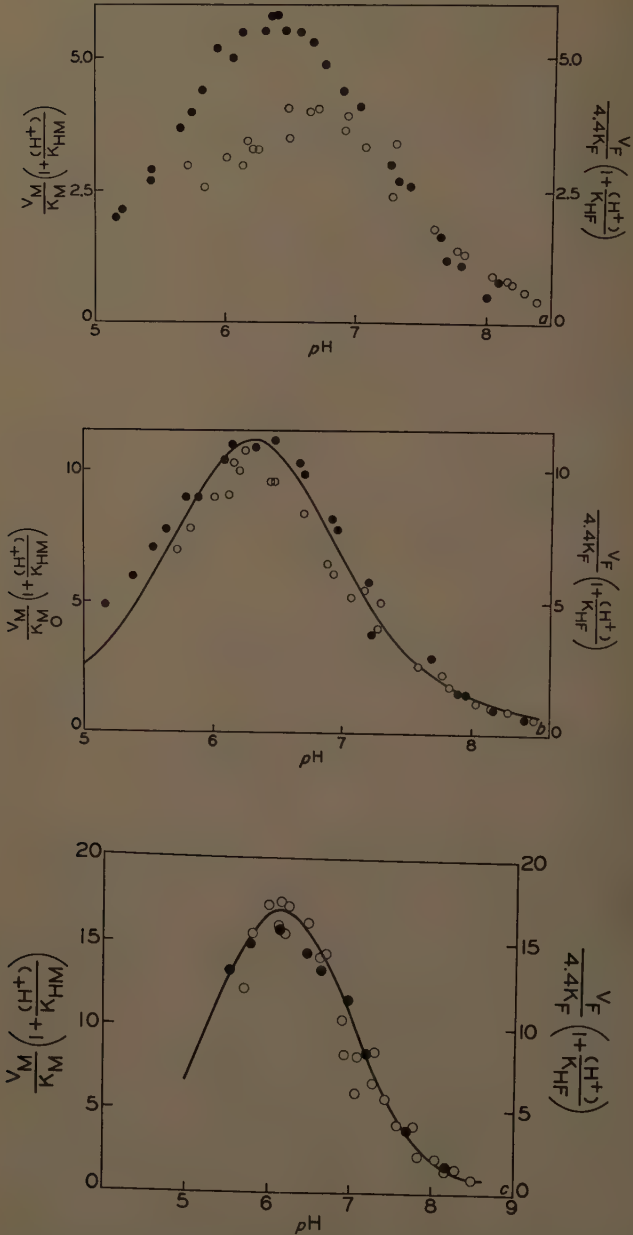


FIGURE 11. Plots of $\frac{V_M \times 10^2}{K_M} \left(1 + \frac{(H^+)}{K_{HM}}\right)$ (left ordinate, open circles) and $\frac{V_F \times 10^2}{4.4 K_F} \left(1 + \frac{(H^+)}{K_{HF}}\right)$ (right ordinate, closed circles) versus pH for fraction A (a), fraction B (b) and pork-heart fumarase (c).

The ionization constants that could be determined are listed in TABLE 1. There are significant differences in the apparent acid ionization constants for the three enzyme-malate complexes, although the values for the apparent basic ionization constants are identical. There are also differences between the ionization constants of the free enzymatic sites of fraction B and pork-heart fumarase.

Discussion

The research that has been described is not sufficient to establish whether the two torula fumarases exist as such in a single yeast cell. It is quite conceivable that the rather long period of extraction at room temperature caused proteolysis such as that responsible for different forms of chymotrypsin.⁸ In

TABLE 1
IONIZATION CONSTANTS OF THE ENZYMES AND ENZYME-SUBSTRATE COMPLEXES

	Fraction A	Fraction B	Pork-heart
pK_{aE}		5.9	5.4
pK_{bE}		6.7	6.9
pK'_{aEM}	7.4	7.1	6.7
pK'_{bEM}	8.7	8.6	8.7
pK'_{aEF}			5.1
pK'_{bEF}			7.1

this case the two enzymes would be merely extraction artifacts. However, even so, the phenomenon would be of interest since pork-heart fumarase tends to be rather unstable and susceptible to denaturation by very mild treatment. It has been shown that when pork-heart enzyme is treated with proteolytic enzymes,⁹ the only result is an immediate loss of activity. Another possibility is the presence of more than one strain of yeast in the culture. Although the yeast was apparently pure when the culture was started (personal communication of Kenneth L. Cartwright*), there may very well have been contamination or mutation at some time in its history. Although the relative proportions of fractions A and B remained constant throughout the period of use of one yeast sample, there was a definite decrease in the proportion of fraction A when the use of a new sample was begun. The variation might have been caused by differences in strain, culture conditions, or drying conditions. Finally, the two enzymes might be derived from subparticles of the cell, such as the mitochondria and cytoplasm. This appears to be responsible for the existence of multiple forms of malic dehydrogenase,¹⁰⁻¹² and could undoubtedly be true in this case.

Whatever the reasons for the presence of two torula fumarases, it is interesting that they exhibit differences in their kinetic behavior. The variations are so subtle that there is no doubt that the enzymes are closely related, but they are nevertheless quite real. Small differences in the structure of the enzyme

* Lake States Yeast and Chemical Division, St. Regis Paper Company.

might easily be responsible for the variations that have been observed between fractions A and B. There is obviously a difference in electric charge between forms A and B since this is the basis for the separation. If better results had been obtained for the V/K plots for fraction A, it would be possible to compare the ionization constants of the active sites of the two enzymes. Once again, however, it is unlikely that the differences, if any, would be sufficiently great to implicate different types of ionizing groups.

Summary

Two molecular forms of fumarase have been isolated from torula yeast and compared in kinetic properties to the previously well-studied pork-heart fumarase. The torula enzymes differ from each other and from the pork enzyme in Michaelis constants and in the dependence of maximum velocity upon pH . There is a striking difference between the enzymes from the two sources in the shape of the plot of maximum velocity with fumarate versus pH .

Acknowledgments

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References

1. ALBERTY, R. A. 1961. *In* The Enzymes. 2nd ed. 5: 53. P. D. Boyer, H. A. Lardy and K. Myrbäck, Eds. Academic Press. New York, N.Y.
2. FRIEDEN, C., R. G. WOLFE, JR. & R. A. ALBERTY. 1957. *J. Am. Chem. Soc.* **79**: 1523.
3. FRIEDEN, C., R. M. BOCK & R. A. ALBERTY. 1954. *J. Am. Chem. Soc.* **76**: 2482.
4. PORATH, J. 1956. *Biochim. et Biophys. Acta.* **22**: 151.
5. EADIE, G. S. 1942. *J. Biol. Chem.* **146**: 85.
6. SCHACHMAN, H. 1959. *In* Ultracentrifugation in Biochemistry. : 86. Academic Press. New York, N.Y.
7. PELLER, L. & R. A. ALBERTY. 1959. *J. Am. Chem. Soc.* **81**: 5907.
8. KUNITZ, M. 1938. *J. Gen. Physiol.* **22**: 207.
9. INAGAKI, M., T. MASUNDA & K. OKUNUKI. 1958. *J. Biochem. (Japan).* **45**: 885.
10. DELBRÜCK, A., E. ZEBE & T. BÜCHER. 1959. *Biochem. Z.* **331**: 273.
11. SIEGEL, L. & S. ENGLARD. 1960. *Biochem. Biophys. Research Comm.* **3**: 253.
12. ENGLARD, S., L. SIEGEL & H. H. BRIEGER. 1960. *Biochem. Biophys. Research Comm.* **3**: 323.

FORMS OF ENZYMES IN INSECT DEVELOPMENT*

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The changes in morphology during insect development that result from endocrine interactions must be reflected in changes in the protein constitution of various tissues. While observing such protein changes by zone electrophoresis in starch gels in *Cecropia* (*Hyalophora cecropia*) and *Cynthia* (*Samia cynthia*) silk moths at different stages of the life cycle, we began to inquire into the catalytic activities of these proteins as a possible clue to their normal function. A number of the blood and tissue proteins were found to possess enzymatic activities. Such activities included esterases, phosphatases, carbohydrases, lipases, sulfatases, and chymotrypsins. All of these appeared in multiple bands in starch-gel electrophoresis (Laufer, 1960). Dehydrogenating enzymes, namely malic dehydrogenase (MDH), α -glycerophosphate dehydrogenase (GPDH), and lactic dehydrogenase (LDH) were also observed to occur in multiple bands.

The present study indicates the existence of isozymes among the hydrolytic and dehydrogenating enzymes that occur in cell-free blood preparations. The stage of development is correlated with the occurrence of changes in the observed proteins and enzymatic activities of the blood. The question of where these multiple molecular forms are elaborated is studied by examining tissues for the release of particular proteins into the blood. Furthermore, physiological and endocrinological factors that influence and regulate some of these enzymes are discussed. Finally, a possible functional significance for some of the multimolecular forms of enzymes is suggested.

Methods and Materials

Animals. The specimens of *Cecropia* and *Cynthia* used in these studies were collected in nature as pupae. Developing adults were obtained by incubating pupae chilled for a sufficiently long period at 4 to 6° C. to permit them to break diapause, a stage of arrested development. *Cynthia* larvae were raised from eggs on *Ailanthus* trees. Blood and tissue extracts were prepared as described previously (Laufer, 1960).

Electrophoretic analysis for enzyme activity. The clear supernatant solutions obtained after centrifugation of samples were analyzed by zone electrophoresis in starch gels and stained for hydrolytic enzyme activity essentially, as described by Hunter and Markert (Laufer, 1960). Dehydrogenase activity was assayed with phenazine methosulfate, neotetrazolium, diphosphopyridine nucleotide (DPN), substrate, and buffer as modified from Nachlas *et al.* 1960 (Laufer, 1961). The only changes have been the replacement of barbital with Tris buffer, 0.02 M, pH of 8.6, in electrophoresis, and 0.2 M for dehydrogenase assays.

Organ culture. A technique destined to be applied more extensively in the

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future for the study of isozymes and for other developmental phenomena is the preparation of organ cultures. Its particular utility here lies in its ability to distinguish sites of synthesis from regions of storage or accumulation. Forty cultures of fat body (200 to 400 mg. of tissue) were incubated in watch glasses containing 0.5 ml. of medium consisting of a mixture of 2 parts tissue culture medium 199 or 1066 from Cappel Products, 4 parts of Ephrussi-Beadle Insect Ringer's solution, and 1 part of insect blood previously heat-treated and centrifuged clear of precipitate. In preliminary experiments, cultures appeared healthy for at least 4 days and, when the heart was included with fat body, it commonly beat for 7 to 10 days when the medium was renewed every 3 or 4 days.

The period of culture was limited to 18 hours to assure maximal viability. The medium was collected and pooled from several cultures, and the proteins precipitated by saturation with ammonium sulfate after preliminary centrifugation. The precipitate was repeatedly washed with ammonium sulfate, and was finally dissolved in a minimum of Ringer's solution. In the case of some preliminary experiments on the incorporation of radioactively labeled amino acids, the precipitate was repeatedly washed with ammonium sulfate until the supernatant was essentially free of radioactivity. The concentrated protein solution was analyzed by means of zone electrophoresis in starch gels and the radioactivity of the proteins was estimated.

Results and Discussion

Qualitative and quantitative changes in proteins and enzymes are observed during the postembryonic development of the giant silk moths. Alterations in activity at different stages of development are shown in representative zymogram patterns (FIGURE 1; see also Laufer, 1960, 1961). Many of the enzymes appear to exist in multiple forms. Here, as in other organisms, there is a stage- and tissue specificity in the patterns observed.

Multiple forms of enzymes. In regard to the esterases: at least 8 different esterase bands were detected in *Cecropia* blood at the beginning of adult development with α -naphthyl butyrate as the substrate and fast blue BB as coupler. At earlier and later stages of development fewer bands were detected.

We were concerned, therefore, lest these numerous bands represent artifacts of the technique, such as differential absorption of one or a few esterases upon the major blood proteins. Of the 8 esterases observed in developing *Cecropia*, 4 migrate along with the major protein fractions, 2 precede the major proteins, and 2 migrate negatively at pH 8.6 and are associated with minor protein constituents (FIGURE 2).

Three of the positively migrating esterases have been separated by the use of a DEAE column by the method of Sober *et al.*, 1956 (FIGURE 3). The others were not recovered from the column in a limited number of experiments. Those that were recovered assumed the same position on reapplication on the starch gel and electrophoresis as they had occupied before, without giving rise to sub-fractions in other positions. Included were (1) the esterase migrating with protein 1, the major blood protein; (2) a slow esterase; and (3) one of the rapidly migrating and active esterases. Two of these esterases tested immunochemically were found to be distinct from one another, as revealed by agar-gel diffu-

sion and histochemical staining of the antigen-antibody precipitates. These esterases retained their enzymatic activity when in combination with their respective antibodies.

The tissue specificity of the esterase patterns was demonstrated by the use of homogenates of individual tissues (FIGURE 4). The tissues such as the midgut and fat body contained esterases with migratory properties similar to components present in the blood. In several instances other enzyme activities were located in the same positions in the zymograms as were occupied by esterases. Identity of mobility in starch electrophoresis does not, of course, indicate an

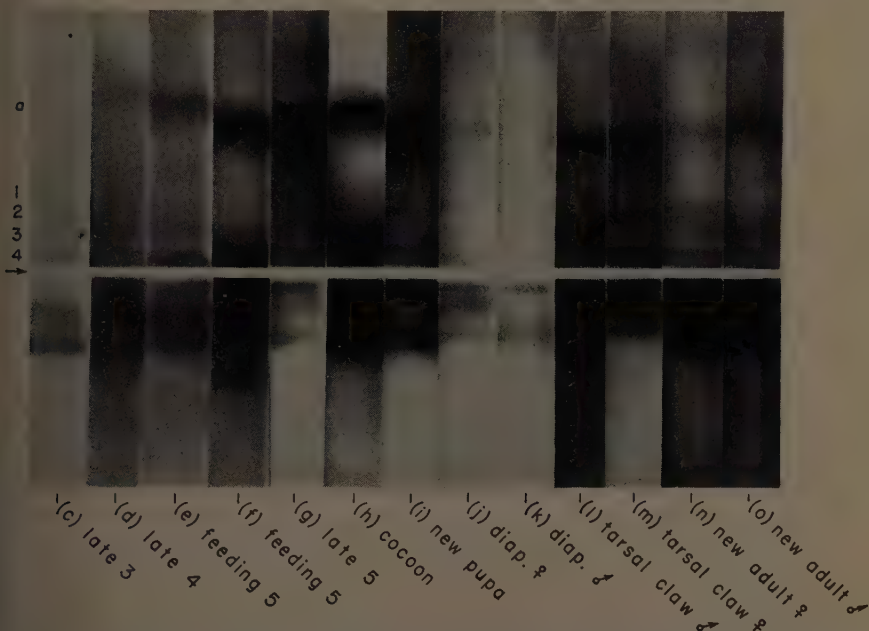


FIGURE 1. Electrophoretic separation of blood protein from several developmental stages of *Cynthia* stained for esterases with α -naphthyl butyrate as the substrate. Enzymatic activity is revealed by coupling the naphthol resulting from the hydrolysis with fast blue BB. The complex, indicating the location of esterase, appears as a black region in the starch. Several esterases are observed. During diapause certain components have diminished activity. Reprinted from Laufer (1960).

identity of the proteins. Further tests, described below, showed that the esterases of the blood were in some cases related to those of the fat body and in others to those of the midgut.

Substrate and inhibitor specificities of the blood esterases were tested (FIGURE 2). One of the more active bands was inhibited by $1 \times 10^{-4} M$ eserine, whereas the remainder retained their original activity. Of the eight esterases observed in *Cecropia* pupae developing to the adult stage, two were preferentially active with indoxyl-butyrate as the substrate; two others were distinguished by their activity toward β -naphthyl caprylate. The esterases would seem to fall into classes or groups, based on substrate specificity, within which they have not been distinguished. These are, therefore, probably isozymes. Examination of the

phosphatases, carbohydrases, chymotrypsins, and others have not progressed far enough even to suggest whether these represent separate enzymes or multi-molecular forms of the same enzyme.

Multiple forms of dehydrogenases. Malic dehydrogenase (MDH) activity was observed in multiple forms in both species of silk moths studied (FIGURES 5 and 6). FIGURE 5 is a schematic summary of assays performed on blood from

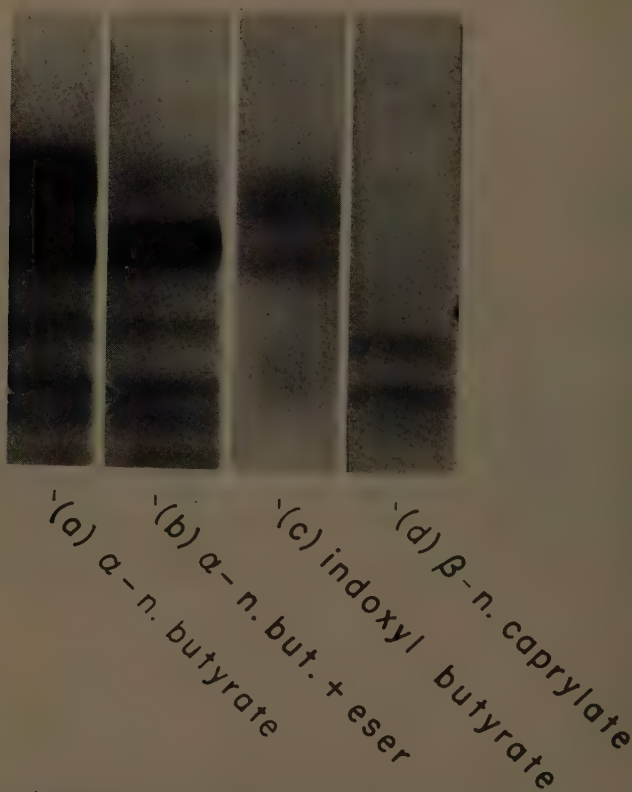


FIGURE 2. As many as eight esterases are found in *Cecropia* developing into adults. The six bands migrating toward the anode are shown (the origin is at the bottom of this figure). The blood sample was incubated in different substrates, or in the presence of the inhibitor eserine. Substrate and inhibitor specificities for some of the esterases are demonstrated. Those that can not be distinguished by such methods are considered to be isozymes. Reprinted from Laufer (1960).

Cynthia taken at several developmental stages. The apparent lack of MDH activity during diapause indicates reduced activity, but should not be considered as demonstrating the absence of this enzyme since formazan production can be detected in blood of diapausing animals both in test-tube reactions and in starch-gel electrophoresis under special conditions of increased reactants and incubation time. The concentration of cytochrome *c* in the wing epithelium of *Cecropia* has also been observed to decrease to undetectable levels (Shappirio and Williams, 1957; Shappirio, 1960), but this again does not necessarily indi-

cate the complete absence of the enzyme, since the intact cytochrome system was most probably functioning (Kurland and Schneiderman, 1959).

As many as three different bands of MDH were resolved in starch gels when *Cecropia* develops into the adult (FIGURE 6). One of these bands is active in the diapausing pupa. A second one is present with low activity, while a third is not detectable at this time. During development the component active in diapause loses most of its activity, and the other two become more active. α -GPDH was observed in multiple forms in both *Cecropia* and *Cynthia* blood (FIGURE 5). Greater activity in both cases occurs during late larval life and during development of the adult than during diapause in the pupa. In an investigation of α -GPDH in insects, Zebe and McShane (1957) found two such different forms: one was soluble and DPN-associated, the second was insoluble, probably localized on the mitochondria, and cytochrome-linked. These proteins would probably be considered distinct enzymes. The dehydrogenases in the present report, however, are all soluble and, therefore, more similar to the first type. The nature of the differences between them is still to be learned.

Identity of enzyme activity of some isozymes may be apparent only in starch gel electrophoresis for, if the enzymes are located in different parts of the cell, or if they function under different physical or chemical conditions, they actually serve different functions.

While the pattern of *Cynthia* blood has revealed multiple forms of LDH, only one band has so far been observed in *Cecropia*. Blood LDHs in *Cynthia* occur in at least three components (FIGURE 5): one that functions throughout development of the pupa and into adulthood, and two others that cease to function during diapause. These observations on dehydrogenases are in essential agreement with the well-known increase of metabolic activity at the onset of development. Other enzymes have been shown to behave similarly (Laufer, 1960, 1961). This change is of particular interest, as it occurs at the time when the molting hormone ecdysone is known to be active and the metabolism is shifted from being predominantly anaerobic (and cyanide insensitive) to one that is both highly aerobic, and cyanide and monoxide sensitive. This suggests that multiple forms of enzymes may participate in metabolism in specific ways. Perhaps certain dehydrogenases are more efficient for a relatively anaerobic metabolism, while others are effective under aerobic conditions. This situation might be comparable to the hemoglobins in man (Thomas *et al.*, 1960), where foetal hemoglobin is synthesized for the relatively anaerobic foetal environment, and the adult form is produced for life in a more aerobic environment. Evidence that isozymic forms of an enzyme may have different substrate affinities lies in the different Michaelis constants obtained for LDHs under a variety of physiological and pathological conditions in man (see for example Hess, 1958; Hill, 1958). While the range of values is not very great, the K_m here represents an average for the mixture of blood isozymes. The K_m s of the individual components may differ considerably from one another. Perhaps the multiple forms of enzymes in some of these cases represent components of alternative metabolic pathways, some of which are always functioning and others operative only during specific active phases of development.

Sources and dispositions of blood enzymes. To determine whether the multi-

Fractions obtained by elution of insect haemolymph from DEAE column

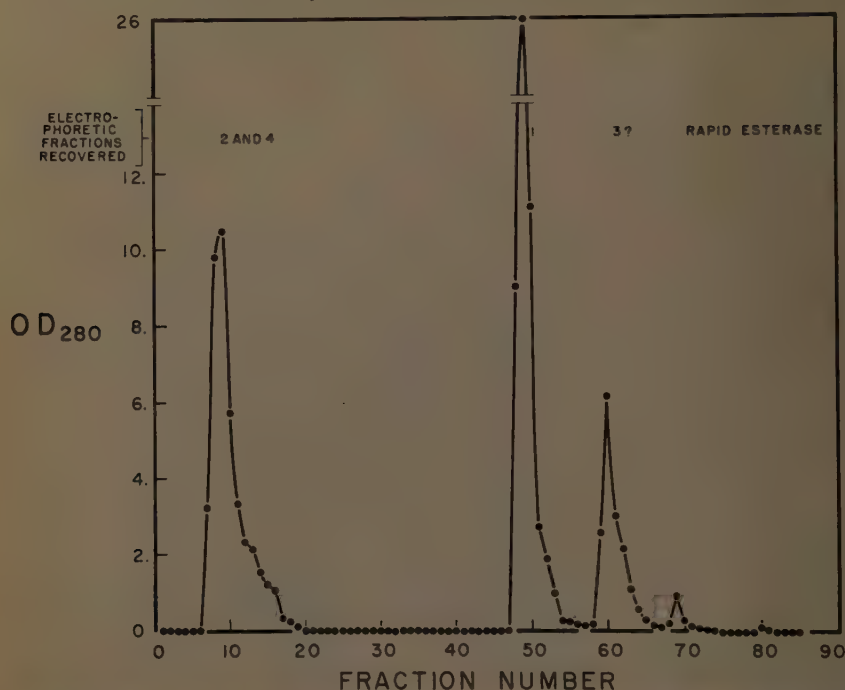


FIGURE 3. Cecropia blood (hemolymph) was separated into several protein fractions by means of a DEAE column. The fractions were then subjected to zone electrophoresis in starch and were also tested for enzymatic and antigenic activity. These experiments showed that no conversion of proteins of one mobility were altered to that of another. Immunochemical diversity of certain fractions and purity of others were also demonstrated.

One representative series of experiments is illustrated. Three major protein peaks and a minor one were recovered from the column. The first peak was inhomogeneous. It contained several proteins, including proteins 2 and 4 as revealed by electrophoresis (the most rapidly migrating major constituent was designated as No. 1). Several antigens were shown to be present in the peak by the method of agar-diffusion immunochemistry (see below).

Tubes 48 to 54, comprising the second peak fractionated by DEAE, contained the most conspicuous electrophoretic component, protein 1. Tubes 58 to 65 contained what was probably protein 3. Tube 69 contained only a small amount of protein but included virtually all of one rapidly migrating blood-esterase which proved to be homogeneous in agar diffusion analysis.

Protein peak number 2 (tubes 48 to 54) contained another esterase as well as phosphatase

molecular forms of enzymes of the blood are incorporated in the formation of adult tissues or cells, an examination of one type of cell was undertaken. The analysis of mature ovarian eggs suggested that blood-born enzymes can be uti-

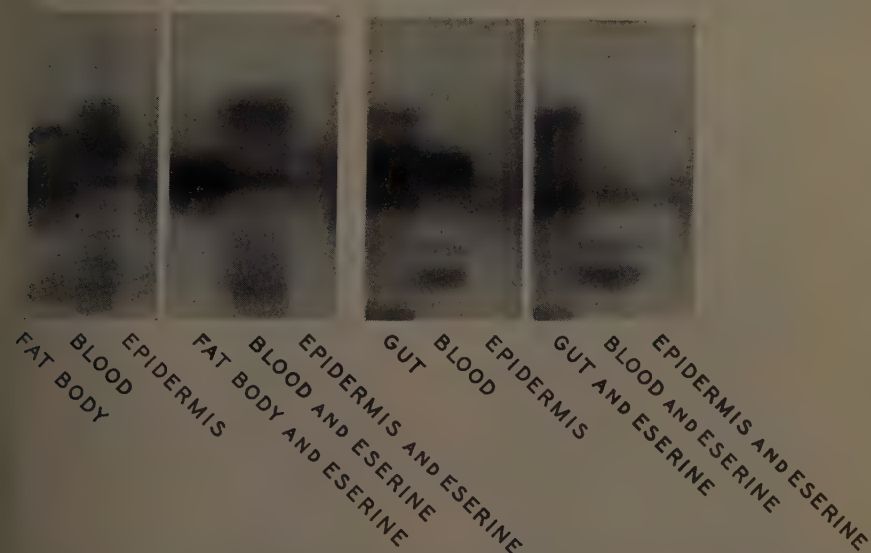


FIGURE 4. Extracts prepared from tissues show that the tissues contain enzymes that produce specific patterns in zone electrophoresis. Here fat body, two blocks to the left, and midgut, the two blocks to the right, are compared with two different blood samples, inserted in the center of each block, and epidermis on the right of each block with and without the addition of the inhibitor eserine. Certain of the esterases are shared by the tissues and blood, others exist in the individual tissues. Experimental procedures revealed that some of the blood esterases are probably contributed by the midgut and others by the fat body.

lized directly in adult structures. Several of the blood enzymes seemed to be represented among the egg contents (Laufer, 1960, 1961). These include two MDHs, two α -GPDHs and one LDH in *Cynthia*. Immunochemical evidence

activity, while the third peak isolated possesses yet another of the esterases. Thus three esterases were separated by this procedure. Immunochemical tests, by means of agar-diffusion (Ouchterlony techniques, see the lower part of this figure; also Laufer, 1960), revealed that these three fractions were immunologically distinct, and that two of the esterases retained their enzymatic activities while in combination with their respective antibody making possible the positive identification of antigen as enzyme. This is illustrated by the immune precipitates that show up in agar as discrete bands wherever antigen and antibody occur in appropriate concentration.

The plates on the left and in the center were photographed by transmitted light. The one on the right is the same as the one in the middle except it was photographed by direct light after esterase staining. The antiserum to *Cecropia* blood was placed in the central reservoir. The reservoir at the upper center of each plate was filled with the unfractionated protein solution as antigen. The other reservoirs were filled with the protein fraction as they were eluted from the column.

The antigens of the plate on the left were all taken from tubes comprising the first peak. In clockwise order, starting from the control reservoir, were samples of tubes 8, 10, 13, 16, and 17. The middle (and right) plate aside from the control antigen, contained samples from tubes 49, 52, 60, 63, and 69. Note the heterogeneity of the first peak eluted from the column. The fractions of the second, third, and fourth peaks have a relatively higher purity. Esterase activity, which was antigenically distinct from that of the other esterases, occurred in the last three peaks. The dark bands in the plate on the lower left represent esterase activity of immune precipitates.

also indicated that blood antigens were utilized specifically and, in some cases, selectively incorporated into eggs (Telfer, 1954, 1960).

Examination of several tissues further suggested that the multiple forms of enzymes might be synthesized by different organs. Experimental analysis

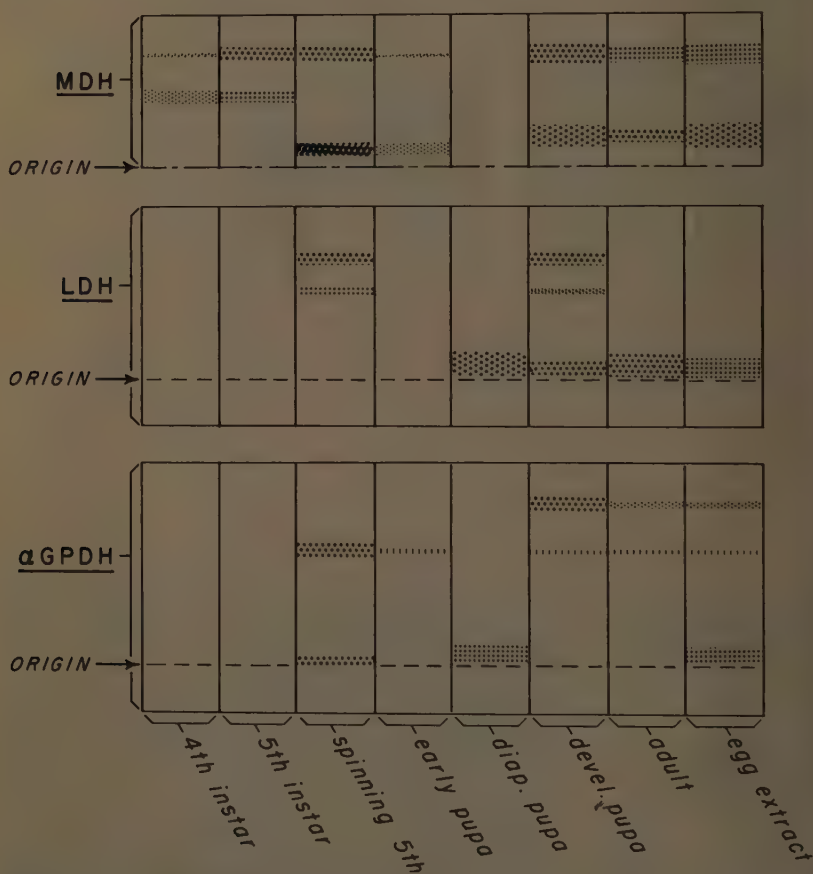


FIGURE 5. Representative patterns of *Cynthia* blood sampled at different stages of development. The enzyme assays show the presence of malic dehydrogenase (MDH), lactic dehydrogenase (LDH) and α -glycerophosphate dehydrogenase (α -GPDH) in multiple forms during the life of *Cynthia*. The absence of a positive test does not necessarily indicate the absence of enzyme, but may imply an activity below the limits of detectability under test conditions.

The MDH activity is generally greater than that of the other two dehydrogenases. During diapause the activities of the dehydrogenases is usually lower than before or after this stage.

showed that the midgut was responsible for the activity of at least two blood esterases, the fat body for that of several others. In the case of the midgut, it could be shown that extirpation of this structure in *Cynthia* pupae prevented the appearance of these esterases, which normally occurred after plasmaphoresis or during development of the adult. With the reimplantation of a midgut, these proteins reappeared in the blood.

Our earlier study revealed that four major protein components present in the blood of female *Cynthia* can also be detected in fat-body homogenates, though at different concentrations than in the blood. Organ cultures revealed that two of these are detectable in culture media of fat body derived from diapausing pupae. One of these proteins corresponded to protein 2 and the other to a

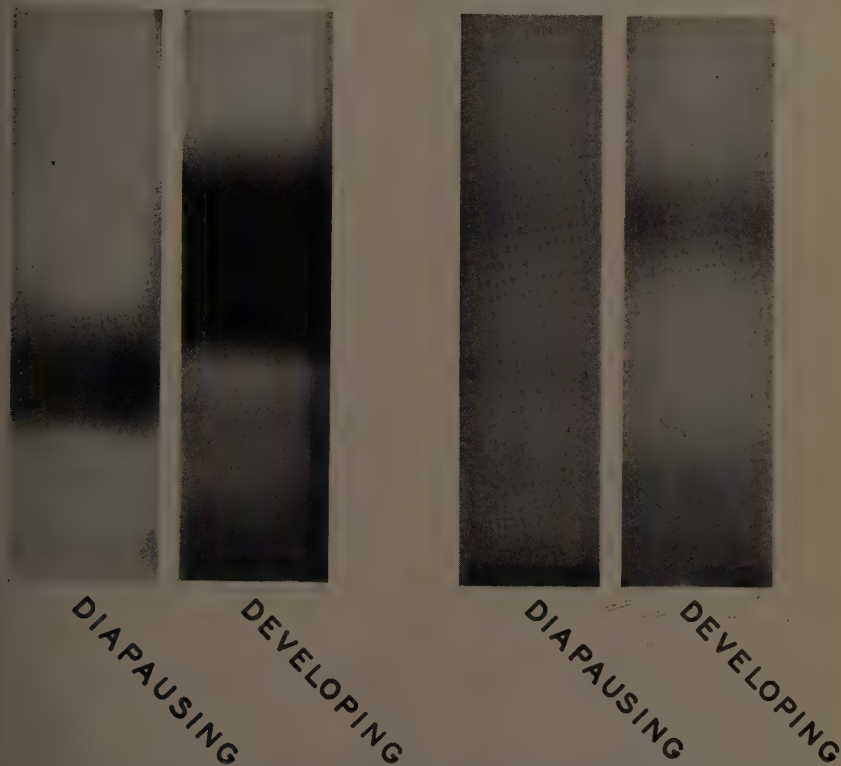


FIGURE 6. (Left) Malic dehydrogenase activity in blood of *Cecropia* sampled during diapause and development of the adult. At least two components of this enzyme are detected in blood of diapausing pupae; while a minimum of three exist in the development of the adult. There is an increase in MDH activity during this development as well as a shift in the relative activities of the component isozymes.

(Right) α -Glycerophosphate dehydrogenase activity of blood from diapausing and developing *Cecropia*. At least two components of this enzyme are detectable during diapause and two during development, but there is an increased activity and a shift in the relative activities of the isozymes during development of the adult.

"female" or "egg" protein. Radioactivity from C^{14} -leucine is relatively high in the region of the negatively migrating esterases associated with wounding (Laufer, unpublished data). Increased radioactivity in this negatively migrating fraction, and increased esterase activity is also observed with normally developing and wounded animals. Of still greater interest was the finding that a MDH that corresponds to a blood enzyme in its mobility was elaborated into the organ culture medium by the fat body. Shigematsu (1958) observed two major proteins corresponding to blood constituents in cultures of fat body de-

rived from 5th instar larva. The 5th instar fat body probably synthesized the major protein, "protein one" at this stage.

While many of the proteins appear to be synthesized *de novo* by the tissues, as indicated by the incorporation of labeled amino acid into proteins, other blood proteins may be released into the blood by lysis of tissue. This seems to be an important factor in the elaboration of blood proteins. Indeed, the hydrolytic enzymes, some of which are proteolytic in nature, may function in the tissue breakdown that is known to occur during normal development and metamorphosis. It would not be surprising if some of the enzymes, particularly those hydrolytic ones with pH optima in the acid range (such as sulphatase, chymotrypsin, and certain esterases) were derived from lysosomes. That lysosomes may function in insect metamorphosis has been suggested by DeDuke (1959).

Extirpation of the brain, which secretes a hormone necessary for further development, resulted in a reduced elaboration of proteins into the blood if the operation was performed prior to the appearance of major blood proteins in the last larval stage (Laufer, 1960).

Conclusions

Most of the hydrolytic enzymes found in insect blood and tissues have distinct specificities toward model substrates, yet these studies have revealed several groups that could not be distinguished except by their electrophoretic mobilities. In the case of the dehydrogenases, which are assayed with physiological substrates, the existence of multimolecular forms with differing electrophoretic mobilities indicates that these enzymes are isozymes. The independent appearance of individual components during specific developmental stages and the tissue specificity of these components suggest that the possibility for artifact is small. A lack of interconversion from one component into another in time, when a mixture is incubated, again argues for the same conclusion, namely that the multiple bands of dehydrogenases observed in insect blood and tissues are indeed isozymes.

The regulation of the tissue and stage specificities of insect enzymes—for example, those associated with the initiation of development—is clearly under hormonal control.

From general genetic considerations one would expect that the chromosomes, in giving expression to the developmental capacities of an insect, determine its enzymatic patterns. Certain insect enzymes such as an esterase (Oppenoorth and van Asperen, 1960), two carbohydrases (Kikawa, 1953), and a dehydrogenase (Hadorn, 1958) have been shown to be genetically determined.

A striking parallel to the multimolecular enzyme patterns in silkworms lies in the structure of polytenic chromosomes in several species of diptera. In these insects the chromosomes have puffing patterns that are specific for each tissue and change during development (Beerman, 1958; Breuer and Pavan, 1955). These puffs, or Balbiani rings, have been interpreted as particularly active functioning sites. Furthermore, Clever and Karlson (1960) reported recently that the puffing pattern of *Chironomus* salivary chromosomes could be altered precociously by the administration of ecdysone, the hormone that normally initiates the development of the insect. It may be, then, as these authors

suggest (see also Schneiderman and Gilbert, 1959), that the insect hormones act in development by regulating enzymes by acting on specific sites of the chromosomes, the isozymes being a particular expression of these developmental interactions.

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References

- BEERMAN, W. 1958. Chromosomal differentiation in insects. *In* Developmental Cytology. : 83-103. (16th Growth Symposium.) Ronald Press. New York, N.Y.
- BREUER, M. E. & C. PAVAN. 1955. Behavior of polytene chromosomes of *Rhynchosciara angela* at different stages of larval development. *Chromosoma*. **7**: 371-386.
- CLEVER, U. & P. KARLSON. 1960. Induktion von puff-veränderungen in den speichel drüsenchromosomen von *Chironomus tentans* durch ecdyson. *Exptl. Cell. Research*. **20**: 623-626.
- DEDUVE, C. 1959. Lysosomes, a new group of cytoplasmic particles. *In* Subcellular Particles. : 128-160. Teru Hyashi, Ed. Ronald Press. New York, N.Y.
- HADORN, E. 1958. Role of genes in developmental processes. *In* The Chemical Basis of Development. : 779-791. W. D. McElroy and B. Glass, Eds. Johns Hopkins Press. Baltimore, Md.
- HESS, B. 1958. DPN-dependent enzymes in serum. *Ann. N.Y. Acad. Sci.* **75**(1): 292-303.
- HILL, B. R. 1958. Further studies of the fractionation of lactic dehydrogenase of blood. *Ann. N.Y. Acad. Sci.* **75**(1): 304-310.
- KIKKAWA, H. 1953. Biochemical genetics of *Bombyx mori* (silkworm). *Advances in Genetics*. **5**: 107-140.
- KURLAND, C. S. & H. A. SCHNEIDERMAN. 1959. The respiratory enzymes of diapausing silkworm pupae: A new interpretation of carbon monoxide-insensitive respiration. *Biol. Bull.* **116**: 136-161.
- LAUFER, H. 1960. Blood proteins in insect development. *Ann. N.Y. Acad. Sci.* **89**(3): 490-515.
- LAUFER, H. 1961. Studies of changes in enzymatic activities of blood proteins in the developing silk moth. 11th Intern. Congr. Entomol. Proceedings Symposium on Insect Chemistry. : 194-200.
- MARKERT, C. L. & F. MØLLER. 1959. Multiple forms of enzymes, tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci.* **45**: 753-763.
- NACHLAS, M. M., S. I. MARGULIES & A. M. SELIGMAN. 1960. A colorimetric method for the estimation of succinic dehydrogenase activity. *J. Biol. Chem.* **235**: 499-503.
- OPPENORTH, F. J. & K. VAN ASPEREN. 1960. Allelic genes in the housefly producing modified enzymes that cause organophosphate resistance. *Science*. **132**: 298-299.
- SCHNEIDERMAN, H. A. & L. I. GILBERT. 1959. The chemistry and physiology of insect growth hormones. *In* Cell Organisms and Milieu. : 157-187. D. Rudnick, Ed. The Ronald Press Co., New York, N.Y.
- SHAPPIRO, D. G. 1960. Oxidative enzymes and the injury metabolism of diapausing Cecropia silkworms. *Ann. N.Y. Acad. Sci.* **89**(3): 537-548.
- SHAPPIRO, D. G. & C. M. WILLIAMS. 1957. The cytochrome system of the Cecropia silkworm. II. Spectrophotometric studies of oxidative enzyme systems in the wing epithelium. *Proc. Roy. Soc. London*. **B147**: 233-246.
- SHIGEMATSU, H. 1958. Synthesis of blood protein by the fat body in the silkworm, *Bombyx mori* L. *Nature*. **182**: 880-882.
- SOBER, H. A., F. J. GUTTER, M. M. WYCKOFF & E. A. PETERSON. 1956. Chromatograph of proteins. II. Fractionation of serum protein on anion-exchange cellulose. *J. Am. Chem. Soc.* **78**: 756-763.
- TELFER, W. H. 1954. Immunological studies of insect metamorphosis II. The role of a sex-limited blood protein in egg formation by the Cecropia silkworm. *J. Gen. Phys.* **37**: 539-558.
- TELFER, W. H. 1960. The selective accumulation of blood proteins by the oocytes of saturniid moths. *Biol. Bull.* **118**: 338-351.
- THOMAS, E. D., H. L. LOCHTE, JR., W. B. GREENOUGH, III & M. WALLS. 1960. *In vitro* synthesis of foetal and adult haemoglobin by foetal haematopoietic tissues. *Nature*. **185**: 397-398.
- ZEBE, E. C. & W. H. MCSHAN. 1957. Lactic and α -glycerophosphate dehydrogenases in insects. *J. Gen. Physiol.* **40**: 779-790.

LOCALIZATION OF SERUM LEUCINE AMINOPEPTIDASE, 5-NUCLEOTIDASE, AND NONSPECIFIC ALKALINE PHOSPHATASE BY STARCH-GEL ELECTROPHORESIS: CLINICAL AND BIOCHEMICAL SIGNIFICANCE IN DISEASE STATES

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Nonspecific alkaline phosphatase (AP), 5-nucleotidase (5N), and leucine aminopeptidase (LAP) are elevated in a wide variety of diseases affecting the pancreas and the hepatobiliary tree.¹⁻³ Nonspecific alkaline phosphatase also rises in osteoblastic bone disease,³ but LAP and 5N are normal.^{1,3} However, high levels of all three enzymes are found in metastatic disease both to bone and liver.

Electrophoresis of AP, 5N, and LAP in normal serum and in serum from patients with diseases involving the liver, biliary tree, pancreas, and bone was performed in order to measure qualitative differences. In normal sera a single prominent peak for both LAP and 5N and two peaks for AP were obtained. In the various diseases, augmentation of the normal peaks and/or additional peaks were noted.

Method

All sera were obtained in the fasting state from normal healthy subjects and from patients on the medical and surgical wards of The New York Hospital, New York, N. Y., whose diagnoses were confirmed either by biopsy, surgical exploration, autopsy, or by their clinical courses.

The study groups for AP, 5N, and LAP are listed in TABLES 1 and 2, and include, in addition to normal subjects and pregnancy, a wide variety of diseases of the pancreas, bone, and hepatobiliary tract.

Serum proteins were separated by zone electrophoresis, according to a modification of the starch-gel method of Smithies.^{4,5} Electrophoresis was carried out at 12° C. at 4.5 v per cm. for 16 hours. The gels were then scored in one-half cm. sections, and each was cut in half along the horizontal plane. One half was stained for protein with a saturated solution of naphthalene black 12B 200 and the other was cut in 0.5 cm. sections that were placed directly into tubes containing the necessary amount of buffered substrates for AP, 5N, and LAP respectively. AP and 5N were determined by a modification of the method of Dixon and Purdom,⁶ using β -glycerophosphate and adenosine-5-phosphate as substrates respectively. LAP was determined by the method of Goldburg and Rutenburg,⁷ utilizing a chromogenic substrate, 1-leucyl- β -naphthylamide hydrochloride. Activity under each peak for AP and LAP was expressed as percentage of the sum total of all 0.5 cm. sections of the gel strips (TABLES 1 and 2).

In later experiments protein was measured in each 0.5 cm. section from starch-gel strips, 4.5 cm. wide. The techniques for staining and cutting of the gel, elution, and protein determination have been described.⁸ On one occasion phosphoethanolamine was used as the substrate instead of β -glycerophosphate in determining the activity of AP in a patient with obstructive jaundice.

Results

Alkaline phosphatase and 5-nucleotidase. Two alkaline phosphatases are noted in sera of normal adults, children, and of patients with elevated AP secondary to bone diseases: Paget's disease, osteomalacia, and metastatic carcinoma. One migrates in the region of the origin to the slow α -2 region, and comprises 2 to 4 per cent of the total AP; the other in the alpha-beta component, specifically in the beta-globulin and fast α -2 region, and accounts for 86 to 96 per cent of the total AP, while in biliary cirrhosis the peak in the region of the origin to the slow α -2 region comprises 23 ± 6.0 per cent, and that in

TABLE 1
ELECTROPHORETIC DISTRIBUTION OF NONSPECIFIC ALKALINE PHOSPHATASE (AP) IN SERUM

Diagnosis	No. studied	No. of peaks of AP found	Mean per cent activity and standard deviation of mean in each peak		
			β Globulin and fast α 2	Origin-to-slow α 2	Prealbumin-2
Normal subjects	10	2	96 ± 3	2 ± 0.05	0
Paget's disease	8	2	92 ± 5	2 ± 0.05	0
Normal children	4	2	86 ± 3	4 ± 0.5	0
Metastatic bone disease	3	2	90 ± 2	3 ± 0.05	0
Hyperthyroidism	2	2	90	3	0
Osteomalacia secondary to malabsorption	4	2	90 ± 4	3.5 ± 1	0
Biliary cirrhosis	8	2	70 ± 5	23 ± 6.0	0
Biliary obstruction secondary to choledocholithiasis and/or carcinoma	4	3	68 ± 7	17 ± 5.0	8 ± 0.5
Infiltrative diseases of the liver: lymphoma, sarcoidosis	4	3	70 ± 8	14 ± 6.0	3 ± 0.05
Laennec's cirrhosis	4	3	72 ± 5	11 ± 3.0	4 ± 0.2
Metastatic liver disease	3	3	66 ± 3	16 ± 2.5	5 ± 0.1

the beta-globulin and fast α -2 region comprises 70.0 ± 6.0 per cent of the total AP (TABLE 1; FIGURE 1).

Three electrophoretic peaks of AP are found in patients with biliary obstruction secondary to choledocholithiasis and/or carcinoma of the pancreas, infiltrative diseases of the liver, Laennec's cirrhosis, and metastatic carcinoma to the liver (FIGURES 2, 3). The major peak, 66 to 72 per cent of the total, resides in the beta-globulin and fast α -2 component; 11 to 23 per cent is in the origin-to-slow α -2 peak, and 3 to 8 per cent is in the prealbumin-2 area. Phosphoethanolamine as substrate did not alter the peaks of AP activity in a patient with obstructive jaundice (FIGURE 4).

In all diseases as well as normals, 5N exhibited a single peak of activity, residing in the region of the origin-to-slow α -2. Specifically, there was no 5N activity in the alpha-beta component. In biliary cirrhosis, biliary obstruction, infiltrative disease of the liver, Laennec's cirrhosis, and metastatic carcinoma

to the liver, the normal peak was augmented, but was unchanged in children and in diseases of bone (FIGURES 1, 2, and 3).

Leucine aminopeptidase. The diseases studied, the number of peaks of LAP and the activity of each peak expressed as percentage of the sum total of all 0.0 cm. sections of the gel are detailed in TABLE 2. The major zone of LAP ac

TABLE 2
ELECTROPHORETIC DISTRIBUTION OF LEUCINE AMINOPEPTIDASE (LAP) IN
SERUM AND BODY FLUIDS

Diagnosis	No. studied	No. of peaks of LAP found	Mean per cent activity and standard deviation of mean in each peak*		
			Postalbumin or fast α 2 to postalbumin	Origin-to- slow α 2	Alpha-2 to β -globulin
Normal subjects	8	1	67.5 \pm 2.2	0	0
Biliary cirrhosis	6	2	68.0 \pm 1.4	11 \pm 2.0	0
Toxic hepatitis secondary to Mitomycin	1	2	55.0	30	0
Acute infectious hepatitis with cholestasis	4	2	48.0 \pm 5.0	26.0 \pm 4.0	0
Acute infectious hepatitis	6	3	57.0 \pm 2.5	9.5 \pm 1.0	14 \pm 0.
Infectious hepatitis (re- covered)	3	1	66.0 \pm 3.4	0	0
Laennec's cirrhosis with acute fatty infiltration	5	3	58.0 \pm 1.8	8 \pm 0.4	20 \pm 0.
Laennec's cirrhosis after re- covery	2	1	68.0	0	0
Lymphoma	1	3	68.0	5	14
Hodgkin's disease	2	3	70.0	5	12
Sarcoidosis	1	3	67.0	5	14
Common duct stone	2	3	45.0	15	15
Carcinoma pancreas with elevated LAP†	5	3	48.0 \pm 0.4	15 \pm 0.5	15 \pm 1.
Carcinoma of head of pan- creas with normal LAP‡	2	1	65.0	0	0
Metastatic carcinoma to liver	4	3	45.0 \pm 2.1	15 \pm 1.1	12 \pm 1.
Infectious mononucleosis	1	3	45	12	20
Pregnancy (at term)	4	2	13.0 \pm 4.5	0	50 \pm 4.
Bile	3	1	80 \pm 1§	0	0
Ascitic fluid	1	1	80§	0	0
Pleural fluid	1	1	82§	0	0

* Mean per cent and standard deviation of the mean per cent when more than two specimens were studied.

† Tumors had either obstructed common bile duct or metastasized to liver.

‡ Tumors neither obstructed common duct nor metastasized to liver.

§ Specimens were diluted 1:1 with normal serum.

tivity coincides exactly with the postalbumin fraction of serum, or lies in the region of the fast alpha-2 and postalbumin fractions.

A single peak of LAP activity was present in the sera of all 8 normal subjects. In 6 specimens, this peak coincided exactly with the postalbumin fraction of serum but, in two instances, it lay in the region of the fast alpha-2 and postalbumin fractions. In 40 pathological sera, there was significant activity in the region between the origin-to-slow alpha-2 and, in 27 of these, a peak of ac

activity was also found in the region of the slow alpha-2 and beta-globulin fraction. This peak was noted as well in 4 pregnant women at term (FIGURE 5).

Three peaks of LAP activity were consistently found in all these cases: acute infectious hepatitis; infiltrative diseases of the liver, that is, lymphosarcoma, sarcoidosis, and Hodgkin's disease; obstructive jaundice, secondary to common bile duct stone or tumor; Laennec's cirrhosis with acute fatty infiltration; and metastatic carcinoma of the liver.

Two peaks of LAP activity were noted in 1 patient with intrahepatic cholest-

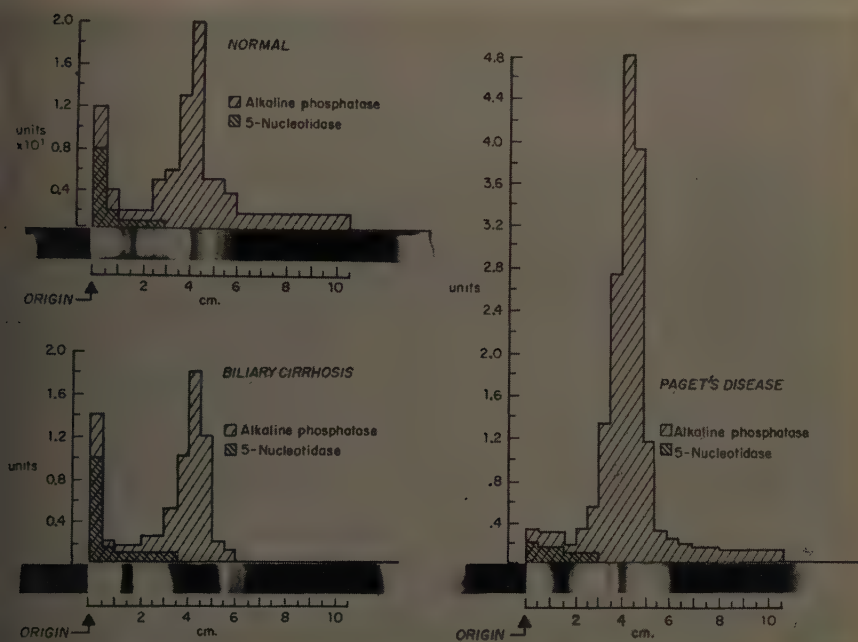


FIGURE 1. Localization of nonspecific alkaline phosphatase (AP) and 5-nucleotidase (5N) by starch-gel electrophoresis. The graph demonstrates peaks of enzyme activities in a normal subject and in patients with biliary cirrhosis and Paget's disease. AP and 5N peaks of similar electrophoretic mobilities were obtained in normal children, osteomalacia, hyperthyroidism, and metastatic bone disease.

sis associated with Mitomycin therapy, in 4 patients with acute infectious hepatitis with intrahepatic cholestasis, and in 6 patients with primary biliary cirrhosis. The predominant peak resides in the postalbumin or fast alpha-2-postalbumin fraction, and the other in the region between the origin and slow alpha-2 fraction (TABLE 2).

Upon complete clinical and biochemical recovery of the 4 patients with acute hepatitis without cholestasis and of the 2 patients with fatty infiltration of the liver, only the single, normal peak in the postalbumin or fast alpha-2 to postalbumin fractions could be seen. A similar observation was made after removal of a common duct stone in 2 patients. The disappearance of the two peaks in these diseases coincided with the fall of serum LAP to normal.

Two peaks of LAP activity were noted in the sera of 4 pregnant women at term. The predominant peak resided in the alpha-2 to beta-globulin fractions and the other in the normal, fast alpha-2 to postalbumin fraction (FIGURE 5).

INFILTRATIVE DISEASE OF THE LIVER (*Lymphosarcoma*)

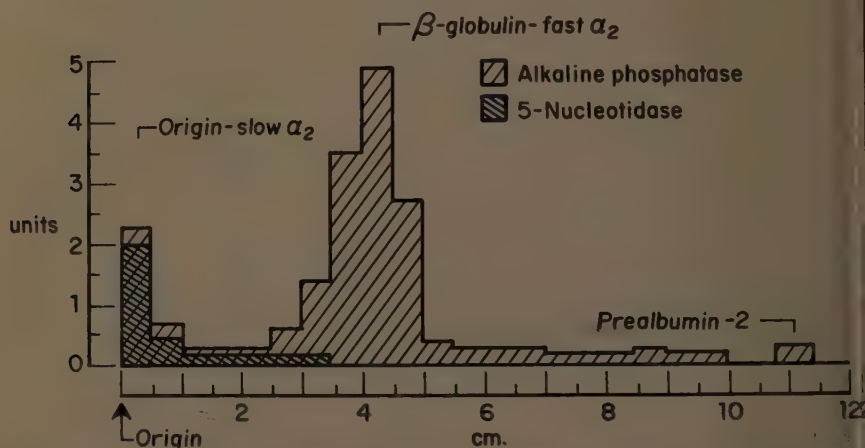


FIGURE 2. Localization of serum AP and 5N in infiltrative disease of the liver (lymphosarcoma).

LAENNEC'S CIRRHOSIS

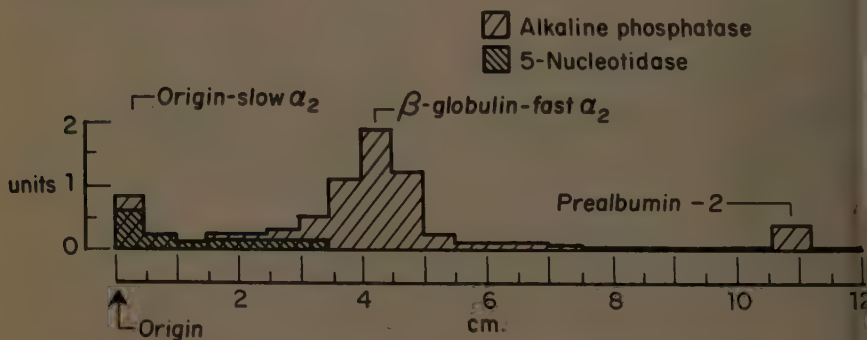


FIGURE 3. Localization of serum AP and 5N in Laennec's cirrhosis.

Discussion

Alkaline phosphatase and 5-nucleotidase. Using paper electrophoresis, Bake and Pellegrino⁹ localized alkaline phosphatase activity in the alpha-2 globulin and, in some cases, observed an additional zone of activity in the beta globulins. Rosenberg,¹⁰ using starch-block electrophoresis, observed that the predominant component was located in the alpha-2 zone, with an additional fraction in the alpha-1 globulin zone. In our studies the major zone of AP activity resides in the alpha-beta component, located principally in the region of the beta globulin or in the beta-globulin to fast alpha-2 region (FIGURES 1 to 4). Lesser peaks

OBSTRUCTIVE JAUNDICE

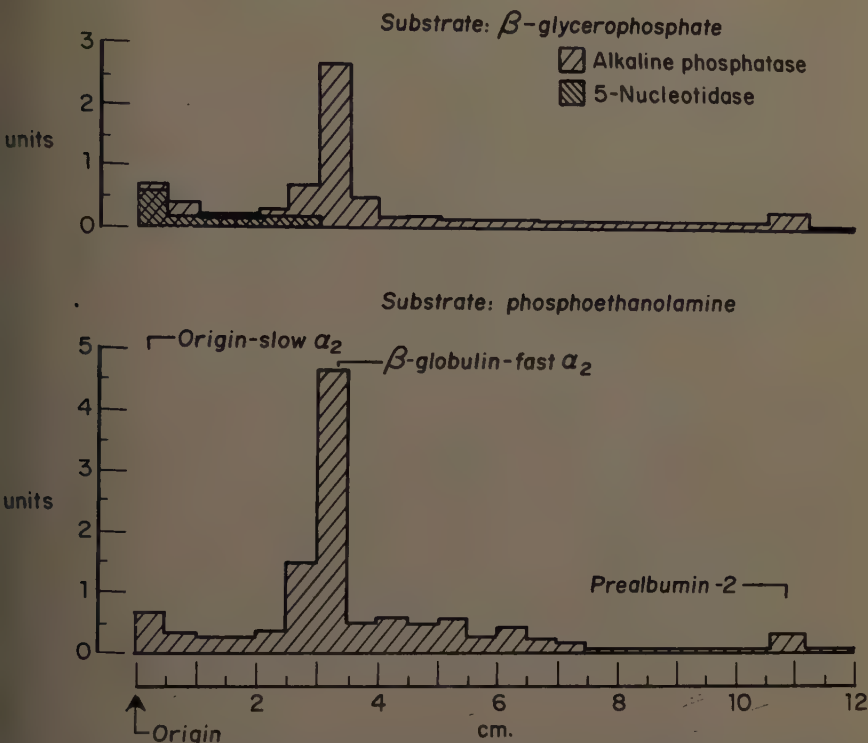


FIGURE 4. Localization of serum AP and 5N in obstructive jaundice: (*upper*) β -glycerophosphate as substrate for AP; (*lower*) phosphoethanolamine as substrate for AP. Peaks of AP activity with either substrate are superimposable.

Localization of serum LAP in pregnancy

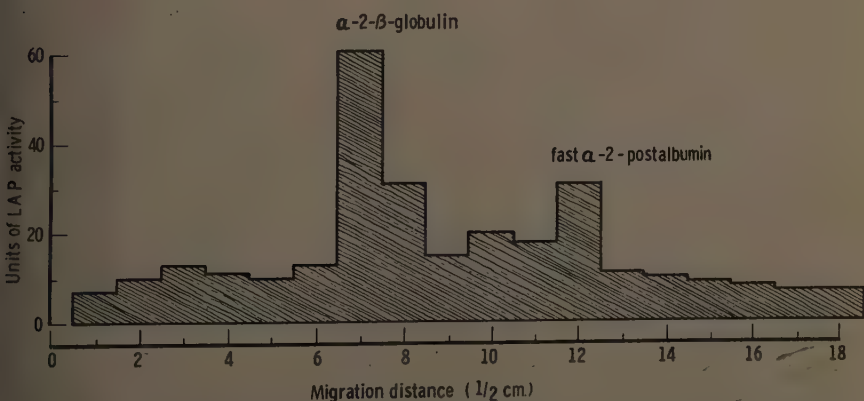


FIGURE 5. Localization of serum leucine aminopeptidase (LAP) in serum from a pregnant female at term.

of activities were found in the origin-to-slow alpha-2 region and in the prealbumin-2 areas. At present we have no evidence that the activity located in these areas corresponds to the activity found in the alpha-1 zone on starch block.

The alkaline phosphatase migrating in the alpha-beta zone is the predominant component of serum AP, regardless of the disease. Because of the similarity of electrophoretic mobility of AP from normal serum and from sera of patients with bone disease, a zone that is devoid of 5N activity, it is likely that bone is the principal source of serum AP. The alkaline phosphatase in the origin-to-slow alpha-2 region and in the prealbumin-2 areas is probably derived from the liver. That the distribution of AP activity was not changed with phosphocreatine as substrate indicates only that AP from liver or intestine can hydrolyze this substrate as well as bone AP¹¹ (FIGURE 4).

We feel that significant information can be gained by determining the electrophoretic mobilities and per cent distribution of serum alkaline phosphatase in many conditions in which the cause of elevated serum AP is not clear; namely, metastatic carcinoma to liver and/or bone, jaundice in patients with bone disease, and infiltrative diseases of the liver. In these diseases, one would expect an augmentation above the normal of both the origin-to-slow alpha-2 component and the alpha-beta component, as well as a peak in the prealbumin-2 area.

Leucine aminopeptidase. With L-leucyl- β -naphthylamide hydrochloride as substrate, LAP of normal serum appears as a single enzyme with an electrophoretic mobility on starch gel that coincides with the postalbumin fraction of the fast alpha-2 to postalbumin fractions of serum. A single peak of LAP similar to normal serum was also demonstrated in bile and bile-serum mixtures (TABLE 2).

The histochemical localization of LAP in bile canaliculi¹² as well as the similarity of the electrophoretic mobility of LAP in bile and serum suggest that these structures are the source of the LAP, which migrates in the postalbumin fraction or the fast alpha-2 and postalbumin fraction.

LAP also appears in the region of the origin-to-slow alpha-2 as well as in the alpha-2 to beta-globulin fractions in a variety of disease states (TABLE 2). The disappearance of these peaks of altered mobility after recovery from acute infectious hepatitis and Laennec's cirrhosis with acute fatty infiltration, and following relief of common bile duct obstruction, suggests the hypothesis that hepatocellular damage and bile stasis produce "enzyme variants" of LAP.

Bile stasis probably accounts for the LAP in the origin-to-slow alpha-2 component, since this peak is found in patients with biliary cirrhosis, toxic hepatitis secondary to Mitomycin, and acute infectious hepatitis with intrahepatic cholestasis.

In pregnancy at term there are two peaks of LAP, the predominant peak lying in the slow alpha-2 to beta-globulin zone. At the present time we have no explanation for this finding, nor can we postulate a source for this peak of increased LAP activity.

The measurement of the electrophoretic mobilities of LAP appears to be useful in several ways: (1) to differentiate "cholangiolitic" hepatitis from acute infectious hepatitis; (2) to follow the clinical course of patients with acute infectious hepatitis, as well as those with Laennec's cirrhosis with acute fatty

infiltration; and (3) to define the degree of biliary cirrhosis, if any, remaining after removal of a common duct stone.

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References

1. KOWLESSAR, O. D., L. J. HAEFFNER, E. M. RILEY & M. H. SLEISINGER. 1961. Comparative study of serum leucine aminoamidase, 5-nucleotidase and non-specific alkaline phosphatase in diseases affecting the pancreas, hepatobiliary tree and bone. *Am. J. Med.*
2. PINEDA, E. P., J. A. GOLDBARG, B. M. BANKS & A. M. RUTENBURG. 1960. Serum leucine aminoamidase in pancreatic and hepatobiliary disease. *Gastroenterology*. **38**: 696.
3. DIXON, T. F. & M. PURDOM. 1954. Serum 5-nucleotidase. *J. Clin. Pathol.* **7**: 341.
4. SMITHIES, O. 1955. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. *Biochem. J.* **61**: 629.
5. PERT, J. H., M. H. SLEISINGER, K. R. WOODS & R. L. ENGLE, JR. 1957. Requirements for optimal resolving power and reproducibility in protein fractionation by starch gel electrophoresis. *Clin. Research. Proc.* **5**: 156.
6. KOWLESSAR, O. D., J. H. PERT, L. J. HAEFFNER & M. H. SLEISINGER. 1959. Localization of 5-nucleotidase and non-specific alkaline phosphatase by starch gel electrophoresis. *Proc. Soc. Exptl. Biol. Med.* **100**: 191.
7. GOLDBARG, J. A. & A. M. RUTENBURG. 1958. Colorimetric determination of leucine aminoamidase in urine and serum of normal subjects and patients with cancer and other diseases. *Cancer*. **11**: 283.
8. KOWLESSAR, O. D., L. J. HAEFFNER & M. H. SLEISINGER. 1960. Localization of leucine aminoamidase in serum and body fluids by starch gel electrophoresis. *J. Clin. Invest.* **39**(4): 671.
9. BAKER, R. W. R. & C. PELLEGRINO. 1954. The separation and detection of serum enzymes by paper electrophoresis. *Scand. J. Clin. Lab. Invest.* **6**: 94.
10. ROSENBERG, I. N. 1959. Zone electrophoretic studies of serum alkaline phosphatase. *J. Clin. Invest.* **38**: 630.
11. MCCANCE, R. A., D. V. I. FAIRWEATHER, A. M. BARRETT & A. B. MORRISON. 1956. Genetic, clinical, biochemical and pathological features of hypophosphatasia. *Quart. J. Med.* **25**(100): 523.
12. NACHLAS, M. N., D. T. CRAWFORD & A. M. SELIGMAN. 1957. Histochemical demonstration of leucine aminoamidase. *J. Histochem. Cytochem.* **5**: 264.

MULTIPLE FORMS OF ESTERASE IN VERTEBRATE BLOOD PLASMA*

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For the past few years my associates and I have been studying the various forms of esterase present in vertebrate plasma.¹ This comparative study has revealed that three types or groups of esterase exist in this material: *arylesterases* (ArE), *aliesterases* (AliE), and *cholinesterases* (ChE). The main characteristic features of these groups are summarized in TABLE 1. The esterases were separated electrophoretically and chromatographically, and each active fraction was studied separately in regard to substrate specificity and susceptibility to selective inhibitors. Some plasmas contain all three types of esterases, others two, and still others only one type.

Evidence will be presented in the following to show that each of the esterase types exists in multiple forms, that each animal species has its own typical set of plasma esterases, and that various forms of each esterase type exist within the same species. In addition, some new features of arylesterases will be reported.

CHOLINESTERASES

The relatively high hydrolysis rate of choline esters compared with that of other esters, and their high sensitivity to eserine are properties sufficiently distinct to regard the plasma cholinesterases as a separate group of esterases. Their sensitivity to certain organophosphorus compounds is shared by the aliesterases, but cholinesterases are easily separated from the latter by electrophoresis or other techniques, and aliesterases do not split choline esters.

Species Specificity

The species specific patterns of cholinesterases are very remarkable (FIGURE 1). The cholinesterases of human, monkey, dog, cat, horse, and guinea-pig plasmas have the specificity pattern of a butyrylcholinesterase. The ruminant plasmas have low cholinesterase activity, and the specificity of these enzymes varies considerably from animal to animal, but in no case was a butyrylcholinesterase detected. Pig plasma cholinesterase has unique properties; it is a butyrylcholinesterase but, in so far as its specificity against various types of ester is concerned, this enzyme differs from all other butyrylcholinesterases studied thus far.^{2,3} An acetylcholinesterase is present in low concentration in the plasma of goat and sheep and in those of all teleostian fish studied. Rat, rabbit, cock, and duck plasmas are characterized by propionylcholinesterases.

Particularly interesting is the esterase present in turtle plasma, as it is highly sensitive to eserine but differs from cholinesterases in hydrolyzing choline esters at a lower rate than noncholine esters. It has been suggested that this esterase is of a phylogenetically intermediate stage between the aliesterases present in

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high concentration in the plasma of lower vertebrates and the cholinesterases of vertebrates belonging to higher orders. It is also of probable significance that the esterase of turtle plasma is a propionylesterase, since such an esterase type may be the most primitive one from a phylogenetic point of view. During the biochemical evolution, the propionyl- and butyrylcholinesterases may have originated from aliesterases, the former being more specialized forms of the latter.

Plasma cholinesterases are to be regarded as a group of esterases the members of which have characteristic but overlapping properties with regard to substrate specificity, sensitivity to inhibitors, and an activity-substrate con-

TABLE 1
CHARACTERISTICS OF THE THREE TYPES OF ESTERASE PRESENT IN
VERTEBRATE PLASMA

	Arylesterases ArE	Aliesterases AliE	Cholinesterases ChE
Other terms proposed	A esterases, aromatic esterases	B esterases, simple es- terases	Nonspecific ChE, pseudo ChE
Specificity: esters hydrolyzed	Ac > Bu	Ac > or < Bu	Ac < (or >) Bu
aromatic esters	+++	+	+
aliphatic esters	-	+++	++
choline esters	-	-	+++
Inhibitors:			
organophosphorus compounds	-*	+++	+++
eserine	-	†	+++
p-hydroxymercuribenzoate	++	-	-
o-iodosobenzoate	-	-	+
La ³⁺	+++†	-	-
EDTA	+++†	-	-
Activator (stabilizer):			
Ca ²⁺	+†	-	-
Electrophoretic mobility (veronal, pH 8.4, I = 0.1), protein region	albumin	α globulin	α - β globulin
Active site (tentatively)	...Cys...	...Ser...	...A ⁻ ...Ser...

* Some hydrolyzed organophosphorus compounds.

† Some are sensitive to 10^{-4} M eserine.

‡ Most of the ArE I.

centration relationship. Cholinesterases with similar enzymatic specificity, for example, the butyrylcholinesterases of certain vertebrates, are also distinguishable molecular types. The butyrylcholinesterases of human, horse, and dog plasmas show very similar substrate specificity. They are, however, different molecular forms because they can be separated from a mixture by physicochemical techniques, for example, electrophoresis (FIGURE 2). The properties of the propionylcholinesterases in rat and rabbit plasmas also have similar specific patterns, but they differ with regard to sensitivity to quaternary ammonium compounds and organophosphorus compounds. Moreover, the owl plasma propionylcholinesterase differs from both rat and rabbit plasma cholinesterases: (1) in the activity-substrate concentration relationship for choline esters (no inhibition by excess acetylcholine or propionylcholine for the avian enzyme); (2) in substrate specificity (high rate of hydrolysis of acetyl-

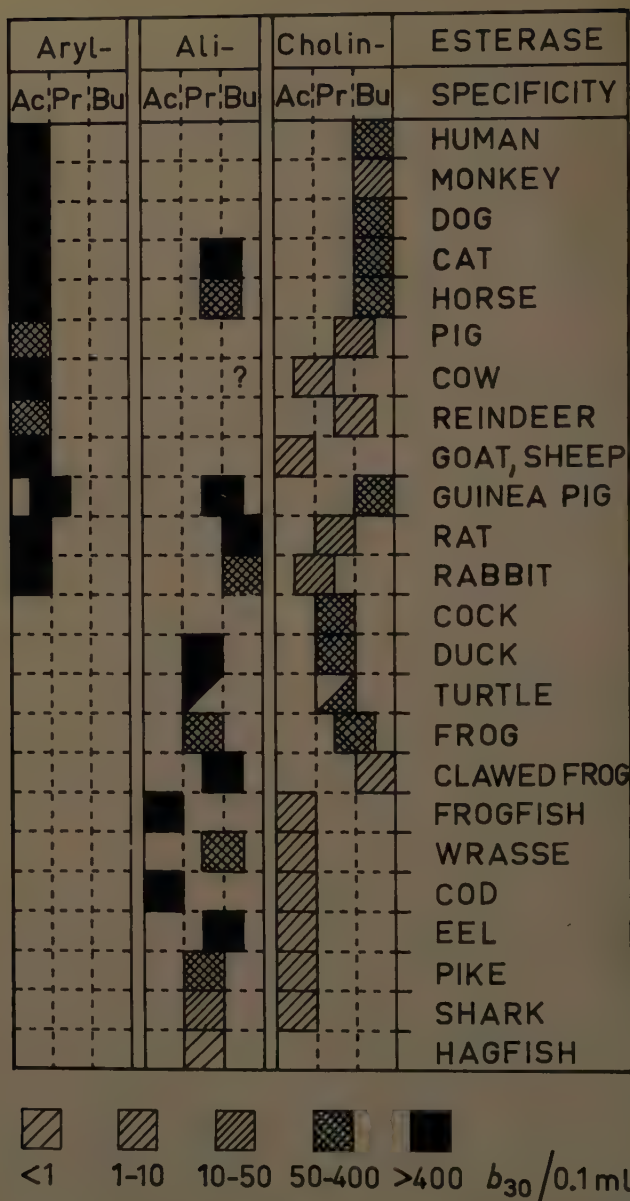


FIGURE 1. Plasma-esterase patterns of vertebrates. Relative esterase specificity based on the acyl radical of the substrate, selectively hydrolyzed by the enzyme in each group: Ac, acetyl; Pr, propionyl; Bu, butyryl. Activity values in b_{30} per 0.1 ml. plasma ($\mu\text{l. CO}_2$ evolved from a bicarbonate- CO_2 buffer in 30 min. at 25°C. , corrections made for the spontaneous substrate hydrolysis) refer to the hydrolysis rates observed with the substrate which is hydrolyzed at the highest rate by each esterase. The properties of the esterase of turtle plasma are those of a propionyl-aliesterase and propionyl-cholinesterase. Reproduced by permission of *Acta Chemica Scandinavica*.

β -methylcholine and various types of noncholine esters); and (3) in a unique pattern of susceptibility to various esterase inhibitors (low-inhibiting effect of certain bis-quaternary ammonium compounds).

It is obvious, therefore, that no clear-cut classification of plasma cholinesterases can be made. The existence of intermediate types between "specific" cholinesterases, such as butyryl-, propionyl-, and acetylcholinesterases, makes it advisable to state the enzyme source in any work with these esterases. Moreover, it is not possible to apply results obtained with the plasma of one species to any other species.

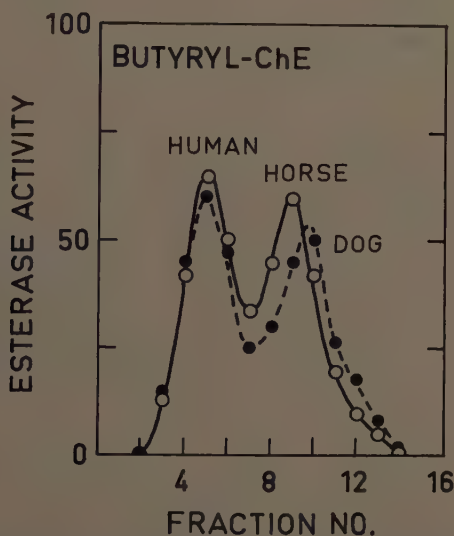


FIGURE 2. Electrophoretic separation of purified butyrylcholinesterases from human, horse, and dog plasmas. Mixtures (3 ml.) of human and horse esterases (○—○), and human and dog esterases (●---●) were applied on cellulose columns (1.5 cm. \times 40 cm.; veronal buffer, pH 8.4, $I = 0.1$; 300 v; 13 hours). Displacement from the column in 1.5 ml. fractions. Esterase activity (b_{30} per 0.4 ml. fractions, Warburg manometric technique) measured with butyrylcholine iodide.

Multiple Forms of Cholinesterase Within the Same Animal Species

Using conventional preparative electrophoresis, we found for most plasmas one single fraction of cholinesterase activity between the α_2 - and β -globulin regions. A number of other investigators obtained the same results with human plasma.¹ In our electrophoresis studies, however, we found cholinesterase fractions of certain plasmas, for example, of cat and dog, that contained more than one active component, exhibiting similar specificity patterns and being equally sensitive to certain esterase inhibitors. Evidence was given recently by other authors, that human plasma also may contain cholinesterase in multiple forms. Heilbronn (personal communication)⁴ demonstrated by indirect means the presence of two enzymes capable of hydrolyzing butyrylcholine, and Berry⁵ from a similar study drew the conclusion that three cholinesterases are present in human serum. Moreover, Dubbs *et al.*⁶ found by starch-gel

electrophoresis of human serum that cholinesterase activity separates into a doublet. In this connection it may be of interest to refer to the observations first made by Kalow,⁷ of an atypical form of cholinesterase in human serum. All these findings suggest the need to continue studies on these problems to find out whether plasma cholinesterase might exist in different molecular forms.

In this connection I mention an observation made recently with butyryl cholinesterase of sow's colostrum and milk.^{2,3} This is the only esterase present in high concentration in this material. The milk electropherogram is charac-

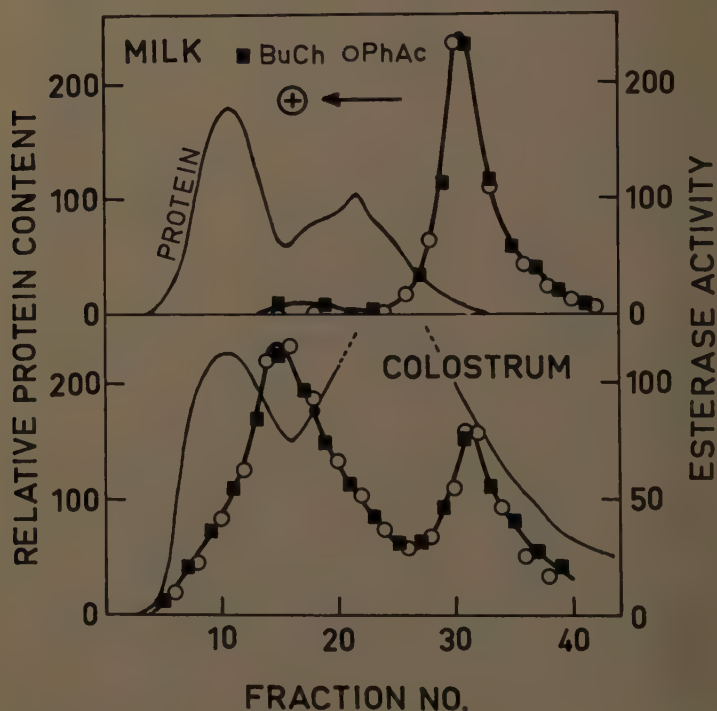


FIGURE 3. Electrophoretic patterns of sow's milk and colostrum obtained with 3.0 ml material in a cellulose column (conditions as indicated with FIGURE 2). Esterase activity ($b_{20}/0.4$ ml. fractions) measured with butyrylcholine iodide (BuCh) and phenyl acetate (PhAc) as indicated.

terized by a sharp esterase peak (FIGURE 3) that moves slower than all major milk protein components. In contrast to mature milk, colostrum gives an electropherogram, in which a second, faster-moving peak is more prominent and the slow-moving peak correspondingly reduced. The two esterase fractions of colostrum have identical properties, in so far as substrate specificity and sensitivity to inhibitors are concerned. The mobility of the faster-moving cholinesterase fraction of colostrum is identical with that of the cholinesterase fraction of pig plasma, and the substrate specificity and other properties are also the same for the two fractions. It is therefore suggested that the faster moving esterase component of sow's colostrum and pig plasma cholinesterase

are identical, and that the electrophoretic mobility of the esterase present in colostrum is lowered during the course of lactation.

Evidence has been presented recently that esterases are localized mainly in the microsomes of animal tissues. Moreover, milk contains microsomes that are derived from mammary tissue and may serve as a vehicle for its butyrylcholinesterase. We therefore suggest that the slow-moving component represents microsomal esterase and the fast-moving one, free enzyme. Recent preliminary experiments actually demonstrated that the esterase activity of sow's milk is associated, partly at least, with the microsomes. The two enzymes are to be regarded as identical molecularly. We are also inclined to believe that this experiment demonstrates that one single enzyme protein can show different electrophoretic mobilities if it moves together with other proteins or cellular constituents, with which it easily combines. Two activities may therefore not necessarily be regarded to be due to two separate molecular forms if only because they can be identified in two separate fractions.

A variation in the specificity pattern of cholinesterases from one tissue to another within the same animal has been reported, but some authors are of the opinion that the specificity pattern of a particular cholinesterase is not organ-specific (literature in Augustinsson⁸). The different opinions as to this specificity problem may be due to the fact that the experiments were carried out with crude preparations in which disturbing proteins, including various types of other hydrolyzing enzymes may be limiting factors.

ALIESTERASES

No clear-cut line of differentiation between various enzymes hydrolyzing aliphatic esters, including choline esters, could be drawn until Richter and Croft in 1942⁹ made the observation that all enzymes classified as cholinesterases were completely inhibited by 10^{-5} *M* eserine, while "simple esterases" were resistant to this inhibitor concentration. "Simple esterases" were those having substrate preference for short-chained fatty acid esters of simple alcohols, and they were differentiated from "lipases" that were more active in hydrolyzing esters of glycerol and fatty acids with long chains. Richter and Croft now introduced the term aliesterase for eserine-resistant esterases, which we have found to be easily separated from the cholinesterases by electrophoresis and in many cases also from the arylesterases. Although the term aliesterase is not adequate for these types of esterases, since some of them hydrolyze aromatic esters at a higher rate than the corresponding aliphatic esters, their use is recommended because the definition by Richter and Croft is still valid. Triglycerides are also hydrolyzed by this esterase type and, since no special esterase hydrolyzing only these esters has been detected thus far in plasma, lipases are included in the group of aliesterases.

Aliesterase is the main esterase in the plasmas of lower vertebrates. It is absent in human, monkey, dog, and pig plasmas, and its presence in ruminant plasmas is questionable. The hydrolysis of aliphatic esters by these plasmas is catalyzed only by cholinesterases, since arylesterase cannot split these esters. Aliesterase has not been detected in cock plasma, but is present in high concentration in fish plasma, and is the only detectable esterase in cyclostome plasma.

Species Specificity and Other Species-Distinguishing Properties

The aliesterases of vertebrate plasma are represented by both acetyl-, propionyl- and butyryl esterases (FIGURE 1); those present in horse, cat, guinea pig, rabbit and rat hydrolyze butyrates and propionates at a higher rate than acetates. The aliesterases of duck, frog, and certain fish species have the characteristics of propionylesterases; in other species of lower vertebrates intermediate types are present. An acetylaliesterase was detected in the plasmas of frogfish and cod. As mentioned, the turtle-plasma esterase displays the characteristics of both an ali- and cholinesterase, including high sensitivity to eserine.

Most aliesterases are highly sensitive to organophosphorus compounds and, when aromatic esters are used as substrates for these esterases, such compounds are the best reagents for differentiating aliesterases from arylesterases, all of which are resistant to organophosphorus compounds. Aliesterases differ from the cholinesterases in being unable to hydrolyze choline esters and being in most cases resistant to 10^{-5} M eserine. The latter agent, however, is not an absolute selective inhibitor of cholinesterases, because the aliesterases of duck, frog, frogfish, and pike are sensitive to eserine in comparatively low concentrations (pI_{50} 5.5 to 4.5), although they do not hydrolyze choline esters. This is another indication of the close relationship existing between the ali- and cholinesterases mentioned earlier.

Multiple Forms of Aliesterase within the Same Animal Species

There is some indication that aliesterases may exist in various forms within the same animal species. In guinea pig plasma, for instance, we detected on electrophoresis two esterases resistant to eserine but sensitive to certain organophosphorus compounds.¹ Both esterase forms were intermediates between a butyryl- and propionylesterase. One of these was found in the α -globulin region, and the other migrated on electrophoresis at a lower rate than cholinesterase (that is, in the β -globulin region). The relative specificity against aliphatic and aromatic esters was somewhat different for the two forms on a quantitative level. Triglycerides were hydrolyzed at a relatively high rate by the fast-moving component which was also alone responsible for the hydrolysis of diacetylmorphine and the carbon analogue of acetylcholine, against which substrates this plasma is highly active.

Multiple forms of aliesterase are probably also present in the plasma of frog, which showed two aliesterase peaks in the electropherogram. Both these esterase fractions probably contained identical esterases, because they showed the same substrate specificity and the same sensitivity to certain inhibitors.

The plasma of eel (*Anguilla anguilla*) is characterized by its unusually high activity in hydrolyzing aromatic esters and triglycerides, especially those of butyric acid. This material seems to be the richest animal source of aliesterase ("lipase") activity ever described. It is 100 times more active than human plasma in hydrolyzing these esters. The main esterase present is a butyryl- or propionylesterase, which is highly sensitive to certain organophosphorus compounds (for example, mipafox and *iso*-OMPA). In addition to this esterase and a cholinesterase present in low concentration, eel plasma contains a second aliesterase (FIGURE 4), which has a lower electrophoretic mobility than

the main aliesterase component and differs from this in hydrolyzing the acetates more rapidly than the butyrates and in being less sensitive to mipafox and *iso*-OMPA. However, eel plasma is highly resistant to eserine, and it may represent a type intermediate between an aliesterase and an arylesterase.

Animal tissues contain, in addition to the esterases described, other types of aliesterase.^{10,11}

ARYLESTERASES

The presence of an aromatic esterase in human plasma was proposed by Mounter and Whittaker.¹² Shortly before this, Aldridge¹³ found two types of serum esterases, A- and B-esterases, hydrolyzing *p*-nitrophenyl esters. In our

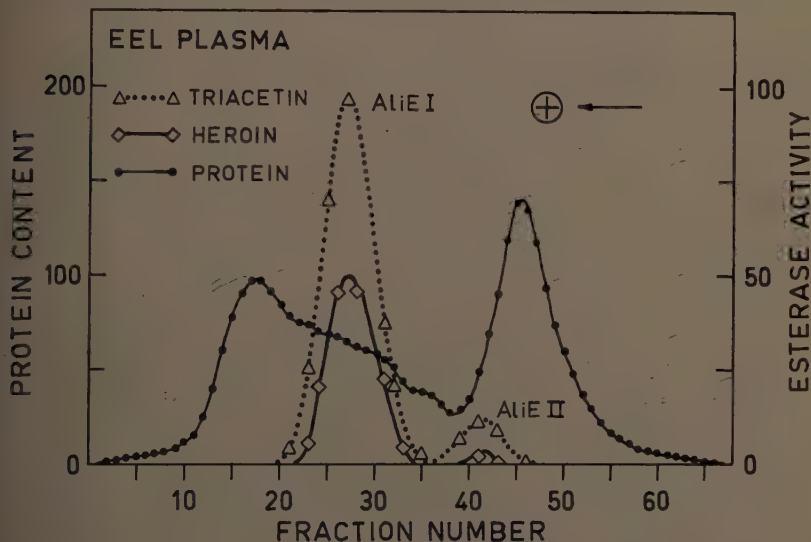


FIGURE 4. Electrophoretic pattern of eel (*Anguilla anguilla*) plasma. Plasma (5 ml.) was applied on a cellulose column (3 cm. \times 40 cm.; 260 v; 30 hours; 2.8 ml. fractions; other conditions as indicated with FIGURE 2). Protein curve based on relative concentration values (Folin color). Esterase activity (b_{20}) obtained with triacetin (0.05 ml. fractions) and heroin (0.1 ml. fractions).

investigations it turned out that among plasma esterases the A-esterase has the highest electrophoretic mobility at pH 8.4, in most cases migrating close to the albumin; it is resistant to a number of organophosphorus compounds and eserine. B-esterase was found to be identical with aliesterase. Since the A-esterases hydrolyzed various aromatic esters, in addition to phenyl esters, the acetates of α - and β -naphthol and indoxyl, but not aliphatic and choline esters, we proposed three years ago the name *arylesterase*¹⁴ for this esterase type. All mammalian plasmas studied so far contain arylesterase in high concentration, but this esterase is absent in all other vertebrate plasmas studied, that is, the plasma of amphibia, reptilia, and fish.

Some General and Common Properties of Arylesterases

Role of calcium. In addition to the inability to hydrolyze aliphatic esters and the resistance to frequently used esterase inhibitors (for example, organo-

phosphorus compounds and carbamates), it is characteristic of several arylesterases that their normal activity and stability are dependent on calcium. During dialysis against distilled water a number of these enzymes lose their activity, which can be restored by the addition of calcium or the dialysate. Maximum activity is obtained when the calcium concentration is 0.1 to 1.0 mM, and no other ions are necessary for normal enzymatic function. The calcium dependence varies on the quantitative level for arylesterases from various species. Sodium ions in high concentration (for example, that of a physiological solution) decrease the activity. Magnesium has the opposite effect to calcium, that is, inhibits esterase activity in concentrations higher than 1.0 mM; this is in contrast to the effect of this metal on cholinesterase and aliesterase, which are generally activated by magnesium. The role of calcium for

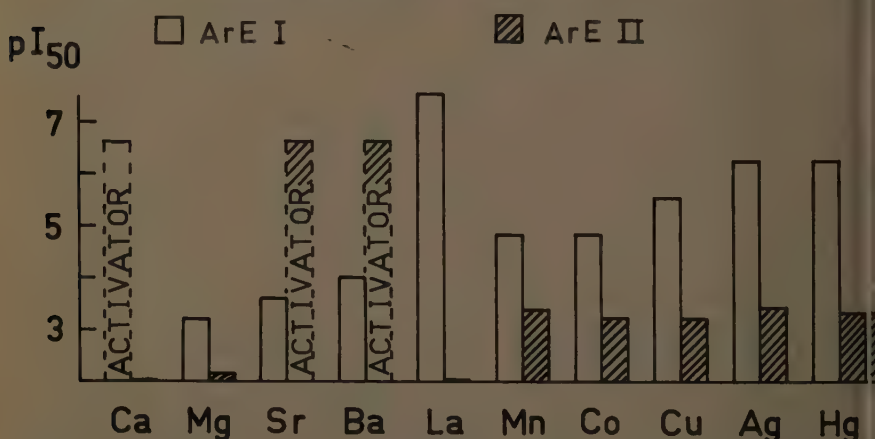


FIGURE 5. Effect of metal ions on arylesterase I (ArE I) and arylesterase II (ArE II, "phosphorylphosphatase") activities of Fraction IV-1 from human serum. Chlorides used except for La and Ag (nitrates); pI_{50} = negative logarithm of molar salt concentration giving 50 per cent inhibition after 50-min. incubation with enzyme before addition of substrate (phenyl acetate for ArE I and dimethylamido-ethoxy-phosphoryl cyanide for ArE II). Esterase determinations made in 40 mM sodium bicarbonate (Warburg technique) at 25°C.

normal function of human serum arylesterase was independently confirmed recently by Erdős *et al.*^{15,16}

Effect of other metallic ions. Cations of heavy metals, as for instance Hg^{2+} , Ag^+ , Cu^{2+} , are strong inhibitors of human serum arylesterase (FIGURE 5). Other inhibiting metallic ions are Co^{2+} and Mn^{2+} . The inhibitory effect of these ions is time-dependent (FIGURE 6), which is in contrast to a recent observation by Erdős *et al.*¹⁵ who used an incubation time of only 5 min., after which approximately 50 per cent of maximum effect (reached after 50 to 60 min. incubation in our experiments) is accomplished under our conditions. Arylesterase inhibited by certain of these metallic ions (for example, Hg^{2+} , Ag^+) can be reactivated by the addition of cysteine or reduced glutation (FIGURE 7; see further below).

Salts of rare earths are highly active inhibitors of certain arylesterases, first observed by Erdős *et al.*¹⁶ with human serum arylesterase. The inhibitory effect of these cations is probably due to their antagonizing calcium. Aryl-

esterases from various species differ greatly in their sensitivity to these trivalent cations. Using lanthanum nitrate in various concentrations, it was observed that two arylersterases are probably present in certain esterase preparations (for example, fraction IV-1 of human serum) differing in sensitivity to La^{3+} (FIGURE 8). Human, dog, horse, and cow plasmas contain one arylersterase that is completely inhibited by $5 \times 10^{-5} M \text{La}^{3+}$; another is inhibited to only 50 per cent by $10^{-4} M \text{La}^{3+}$. It was also observed that the plasma of newborn calves contain only the La-resistant arylersterase, the sensitive component being produced after birth and reaching adult levels at the age of 6 to 7 weeks (FIGURE 8). The arylersterase activity of pig plasma is due entirely, or almost entirely, to the arylersterase type, which is resistant to low concentrations of La^{3+} . These differences between various types of arylersterase were not seen

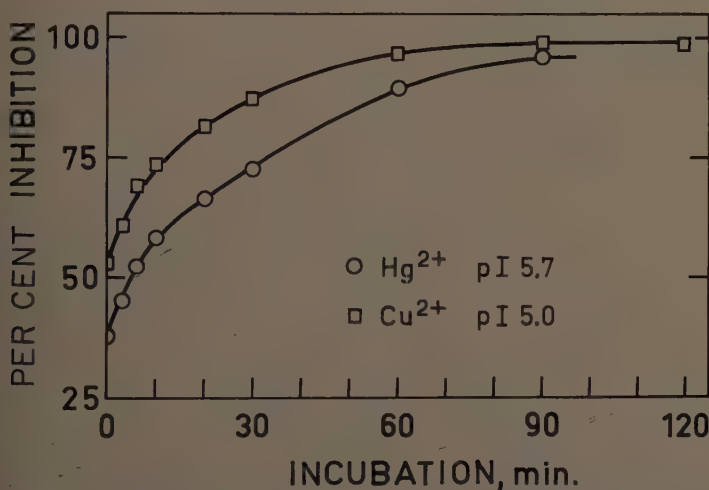


FIGURE 6. Effect of Hg^{2+} and Cu^{2+} on arylersterase activity of purified enzyme from human serum at various incubation periods. Conditions for esterase determinations as indicated with FIGURE 5.

by Erdős *et al.*^{15,16} who used another technique (spectrophotometric) for esterase assay and a different dilution of serum.

Effect of SH reagents. Certain SH reagents are powerful inhibitors of most arylersterases studied, but species differences exist in this respect. In addition to the metal ions mentioned, these agents are useful selective inhibitors of the esterases, since both ali- and cholinesterases are resistant to those concentrations of the SH reagents that give at least 50 per cent inhibition of arylersterase activity (TABLE 2). The most useful inhibitors are those leading to mercaptide formation, for example, *p*-hydroxymercuribenzoate (and *p*-chloromercuribenzoate). Other SH reagents, as for instance the alkylating agents iodoacetamide and *o*-iodosobenzoate, have in most cases the same low-inhibiting effect on all types of plasma esterase. Aliesterases are especially resistant to the SH reagents tested; cholinesterases seem to be more sensitive to *o*-iodosobenzoate than aryl- and aliesterases.

The high sensitivity of arylersterases to mercaptide-forming agents suggests

that —SH groups are required for the activity of arylesterases. This hypothesis is strengthened by the observation that inhibition by these agents is reversed by cysteine. As a tentative model for the mechanism of action we suggest that the enzymatic hydrolysis involves the formation of a thioester intermediate from the enzyme SH and the acyl moiety of the substrate. An —SH group

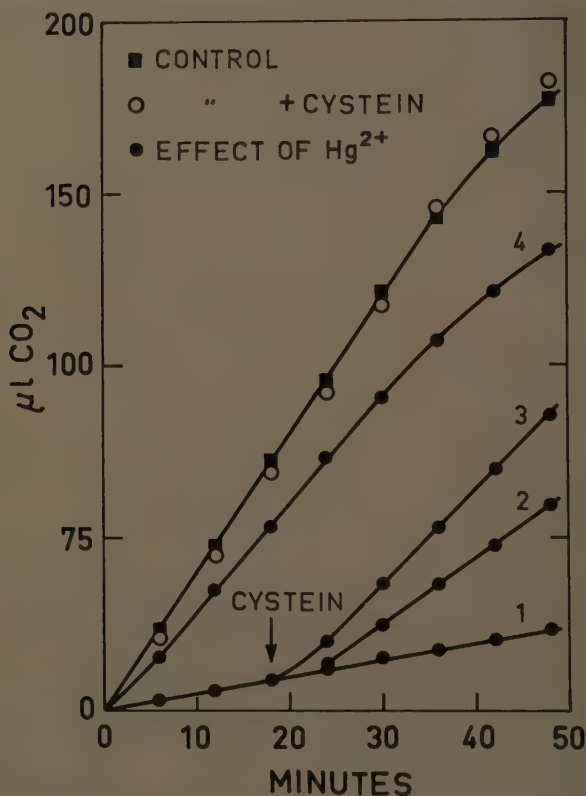


FIGURE 7. Inhibition of arylesterase activity of purified enzyme from human serum by Hg^{2+} and its reversibility by cysteine. Conditions for esterase determinations as indicated with FIGURE 5. (1) Enzyme incubated 50 min. with $2.0 \times 10^{-6} M$ HgCl_2 before addition of substrate. (2 and 3) Cysteine (0.1 mM and 1.0 mM, respectively) added 18 min. after start of esterase determination (that is, after addition of substrate). (4) Enzyme incubated 50 min. with a mixture of $2.0 \times 10^{-6} M$ HgCl_2 and 1.0 mM cysteine before addition of substrate. Cysteine had the same reactivating effect on arylesterase inhibited by CuCl_2 and *p*-hydroxymercuribenzoate respectively.

of cysteine may play the same role for arylesterase action as does the serine hydroxyl for ali- and cholinesterases. This mechanism may also explain why some organophosphorus compounds can be used as substrate for certain arylesterases instead of inhibiting them. It is known that a phosphoryl-S bond is much more easily hydrolyzed spontaneously in the presence of water than is the corresponding phosphoryl-O bond.¹⁷ Thus, in contrast to ali- and cholinesterases, phosphorylation, and probably also carbamoylation¹⁸⁻²⁰ of arylesterases are reversible reactions.

Sex hormones as a possible factor controlling arylesterase biosynthesis. A great interindividual variation in plasma esterase activity is characteristic for several vertebrate species. This variation in activity of human plasma cholinesterase

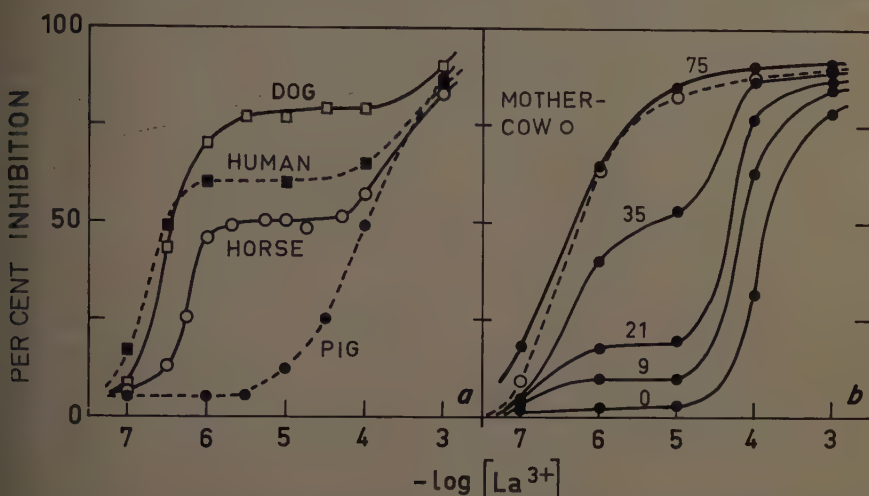


FIGURE 8. Effect of $\text{La}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ on arylesterase activity of whole plasma. Experimental conditions as indicated with FIGURE 5. (a) Various plasmas. (b) Cow and her calf; numbers refer to age in days of the calf.

TABLE 2
EFFECT OF SH REAGENTS ON PLASMA ESTERASES

Esterase type	Animal species	Substrate	pI_{50}^*		
			<i>p</i> -Hydroxy-mercuribenzoate	Iodoacetamide	<i>o</i> -Iodobenzoate
Arylesterase	Man	Phenyl acetate	6.2	<3	<3
Arylesterase	Horse	Phenyl acetate	6	—	—
Arylesterase	Cow	Phenyl acetate	4	<3	<3
Arylesterase	Pig	Phenyl acetate	5.2	<3	<3
Arylesterase	Rabbit	Phenyl acetate	5.5	—	—
Alisterase	Horse	Tripropionin	<3	<3	<3
Alisterase	Guinea pig	Tripropionin	<3	<3	<3
Alisterase	Duck	Tripropionin	<3	<3	<3
Cholinesterase	Man	Butyrylcholine	<3	<3	4.8
Cholinesterase	Horse	Butyrylcholine	<3	<3	5
Cholinesterase	Rabbit	Propionylcholine	<3	<3	<3

* Negative logarithm of molar inhibitor concentration giving 50 per cent inhibition after 50-min. incubation of enzyme with the inhibitor.

has been known for a long time. During studies on arylesterases we found the interindividual variation in the activity of this esterase type still more pronounced for certain animal species, for example, pig, rabbit, rat. In so far as adult normal humans are concerned, this variation was also found to be appreciable, activity values ranging from 80 to 250. In this preliminary investigation there were no sex differences (50 humans of both sexes).

In an extensive series of test crosses, including back-cross tests with pigs, we have presented evidence that arylesterase in pig plasma is genetically controlled.²¹⁻²³ The various phenotypic activity levels, measured when the pigs are 50 to 100 days old, are produced by a set of multiple alleles (a , A_1 , A_2 , A_3 , A_4), each determining a plasma arylesterase activity of 0, 25, 50, 75, and 100, respectively. The distinguishable phenotypes are the manifestation of an additive effect of a pair of these alleles, for example:

Genotype	Phenotype
aa	0
aA_2	50
A_1A_1	50
A_2A_3	125

The plasma of newborn pigs has no arylesterase activity, irrespective of high or medium activity in the plasma of their parents. Differentiation into groups of various esterase activities begins on the second to fourth day of life, adult values being reached at the age of about 50 days. We concluded that the biosynthesis of pig plasma arylesterase after birth is genetically determined according to the theory described. In cows, too, one of the two arylesterases present (ArE I) is absent at the time of birth and is produced during the first weeks of life (see above).

In our genetic studies with pigs, the male piglets were castrated at the age of three to four weeks. However, certain male piglets were selected for use in later cross tests. With these uncastrated boars we observed that the arylesterase activity after maturity was much lower than before sexual maturity. For instance, it was demonstrated with a boar of esterase activity 150 before maturity that the activity gradually decreased with increasing age to a value of about 30, and that this low phenotypic activity was then fairly constant. This phenomenon was found to be common to all mature boars, the low phenotypic esterase activity of which had no influence on the expected segregation in the progeny. Evidence has now been presented that this decrease in arylesterase activity is due to the influence of male sex hormones on the biosynthesis of active protein. When a mature boar with a low phenotypic activity of 30 was castrated, the arylesterase activity increased gradually and had reached, after three weeks, a value (130 to 140) close to that found before maturity (150). Moreover, this high activity could again be reduced to the low value when 100 mg. of testosterone was administered daily intramuscularly; the low value was reached about three weeks after starting hormone treatment.

In contrast to mature boars, the sows show small variations in plasma arylesterase activity. In some cases sows had higher esterase activity when sexually matured than two to three months before maturity. We observed that the activity was slightly higher briefly before partus, decreased below the normal value at the time of delivery, increased during the two to three weeks after delivery and then again decreased to normal. It is possible that these variations in arylesterase activity are partly due to changes in blood plasma volume during pregnancy. For sows, too, we believe, however, that arylesterase biosynthesis is influenced by sex hormones. This problem is under investigation.

Turnover rate of plasma arylesterase in pigs. The turnover rate of plasma arylesterase was determined in some experiments with pairs of pigs (of the same litter), using an exchange transfusion technique by establishing venous and arterial communications between both animals and waiting for complete blood equilibration. The results of one of these experiments are illustrated in FIGURE 9. Exchange transfusion was performed with pig A of arylesterase phenotype 125 (genotype A_2A_3) and pig B from the same litter with activity O (genotype aa). Blood samples were taken for analysis immediately before and after

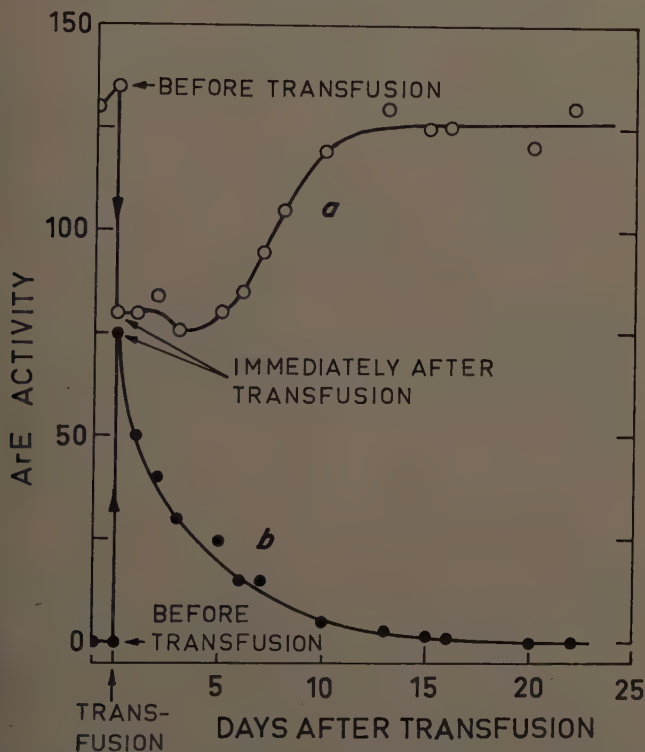


FIGURE 9. Arylesterase activity after exchange transfusion by venous and arterial communications between pig *a* and pig *b* (from the same litter). Arylesterase phenotypes was 125 and 0 (zero) for *a* and *b* respectively. (In collaboration with B. Olsson and H. Å. Åsheim, the Royal Veterinary College, Stockholm, Sweden.)

transfusion, and then regularly during a period of about three weeks. After transfusion the arylesterase activity in both animals was 75 to 80. In pig B the activity then decreased exponentially and, from the straight line obtained by the semilogarithmic plot of esterase activity against time, the half time of arylesterase turned out to be 2.5 days. This value is of the same magnitude as that recently found for certain plasma proteins in mammals.²⁴ It could be stated from separate experiments *in vitro* that pig B did not contain antibodies responsible for the decrease in enzyme activity.

The arylesterase activity of pig A was not significantly altered during the first five days after transfusion, due to the counteraction of esterase destruction

and esterase formation. The activity then increased and had reached the normal value of this animal after about another 10-day period. Details of this and similar experiments will be published in due course in collaboration with B. Olsson and H. Å. Åsheim.

Species Specificity

With few exceptions, plasma arylesterases are *acetylerases*. In all cases studied aromatic butyrates (phenyl and naphthyl) were hydrolyzed at a much lower rate than the corresponding acetates. The species differences in substrate specificity of these enzymes do not seem to be as great as are characteristic for ali- and cholinesterases. *Para*-substitution in phenyl acetate with a nitro group usually greatly decreases the enzymatic hydrolysis rate. For certain plasma arylesterases such a substitution in the aromatic ring increases the hydrolysis rate for pig esterase up to eight times. The arylesterase of guinea pig plasma differs from most other arylesterases in hydrolyzing phenyl propionate at a higher rate than the corresponding acetate.

As mentioned previously, arylesterases from various species differ in their sensitivity to various metal cations. For instance, certain of these esterases are highly sensitive to La^{3+} , others are resistant to high concentrations of this cation (for example, pig arylesterase). The activating and stabilizing effects of calcium and the heat stability are also different from species to species. In certain plasmas, both types of arylesterase (ArE I and ArE II) are present.

Vertebrate tissues probably contain still other types of arylesterase.²⁵

Multiple Forms of Arylesterase Within the Same Animal Species

Most mammalian plasmas contain arylesterase in various forms. Two or more active fractions were obtained on electrophoresis of human, reindeer, dog, rabbit, and rat plasmas. These forms showed similar substrate specificity or differed slightly in hydrolysis rates of various aromatic esters. In so far as sensitivity to certain metal cations is concerned, the differences were more pronounced. Human plasma, for instance, contains at least two arylesterases with more or less identical substrate specificity patterns, but they differ, for instance, in respect to sensitivity to La^{3+} . These two esterases showed also different heat stability, one (ArE I) being inactivated after heating at 56° C. for 30 min. (also found by G. Lundblad; personal communication).

Some but not all arylesterases are responsible for the hydrolysis of aromatic phosphates, for example, *p*-nitrophenyl phosphate and its diethyl derivative (paraoxon). In contrast to alkaline phosphatase, arylesterase hydrolyzes the unsubstituted *p*-nitrophenyl phosphate at a higher rate than the diethyl derivative. In addition, the hydrolysis by blood plasma of other organophosphorus compounds, for example, diisopropyl-phosphoryl fluoride (DFP), dimethyl-amido-ethoxy-phosphoryl cyanide (Tabun), and tetraethylpyrophosphate (TEPP) is in some cases due to arylesterase.¹⁸ Certain arylesterases are also responsible for the hydrolysis of dimethylcarbamoyl fluoride¹⁸ and other *N*-substituted carbamates.^{19,20} Thus these phosphates and carbamates can be used as substrates for one of the arylesterases (ArE II) in human plasma, but not for the other (ArE I). ArE II seems to be identical with the phosphoryl-

phosphatase described recently²⁶ and has been shown to be the lanthanum resistant arylesterase.

We suggest that the hydrolysis of organophosphorus compounds and carbamates by arylesterase can be explained analogously to the reaction of other types of esterase with these agents. The intermediate phosphoryl and carbamoyl derivatives produced with arylesterase are easily broken down by reaction with water, but those formed with aliesterases and cholinesterases are not susceptible to this reaction.

CONCLUSIONS

There exist several types of enzymes that exhibit esterase activity. As far as the catalytic reaction is concerned, the same general mechanism is probably involved. This mechanism of action involves the production of an intermediate acyl derivative which can react with a variety of acyl acceptors (for example, water, alcohol, hydroxylamine, and oximes). The esterase inhibition can be similarly explained, for instance, when the acyl enzyme derivative does not break down by reaction with water. In addition, the hydrolysis of and esterase inhibition by certain phosphoryl, carbamoyl, and sulfonyl derivatives can be explained by an analogous mechanism.

Substantial evidence has been presented to show further that the catalytic reaction involves an interaction between a nucleophilic imidazole group and one of the serine residues in the enzyme molecule. The role of the former is still controversial, but an imidazole derivative may well exhibit a coenzymatic activity in these processes. In certain cases it was also possible to demonstrate the sequences of three to four amino acids adjacent to the serine residue. As a tentative model we have proposed that cysteine replaces serine in arylesterases.

If it is now suggested that this amino-acid sequence is actually characteristic of the active surface of the enzyme in question, the sequence of the many other amino acids in the enzyme molecule may vary without altering the specific activity of a protein. In this way a family of closely related molecular types of esterase may be built up, exhibiting identical substrate specificity but differing in physicochemical properties that are of minor or no importance for the substrate-enzyme reaction. The proposed term isozymes²⁷ should therefore be restricted to enzymes, the molecular structures of which differ only in those parts of the molecules that are not directly involved in the enzymatic reaction.

REFERENCES

1. AUGUSTINSSON, K.-B. 1959. *Acta Chem. Scand.* **13**: 571, 1081, 1097.
2. AUGUSTINSSON, K.-B. 1958. *Acta Chem. Scand.* **12**: 1150.
3. AUGUSTINSSON, K.-B. & B. OLSSON. 1959. *Biochem. J.* **71**: 477.
4. HEILBRONN, E. 1958. *Acta Chem. Scand.* **12**: 1879.
5. BERRY, W. K. 1960. *Biochim. et Biophys. Acta.* **39**: 346.
6. DUBBS, C. A., C. VIVONIA & J. M. HILBURN. 1960. *Science*, **131**: 1529.
7. KALOW, W. & K. GENEST. 1957. *Can. J. Biochem. Physiol.* **35**: 339.
8. AUGUSTINSSON, K.-B. 1961. *Classification and Comparative Enzymology of the Cholinesterases*. Heffter-Heubner Handb. exptl. Pharmakol. Anticholinesterase Agents (ed. G. B. Koelle). Springer Verlag.
9. RICHTER, D. & P. G. CROFT. 1942. *Biochem. J.* **36**: 746.
10. ALDRIDGE, W. N. 1954. *Biochem. J.* **57**: 692.

11. BERGMANN, F., R. SEGAL & S. RIMON. 1957. *Biochem. J.* **67**: 481; *idem.* 1958. *ibid.* **68**: 493.
12. MOUNTER, L. A. & V. P. WHITTAKER. 1953. *Biochem. J.* **54**: 551.
13. ALDRIDGE, W. N. 1953. *Biochem. J.* **53**: 110.
14. AUGUSTINSSON, K.-B. 1958. *Nature*. **181**: 1786.
15. ERDÖS, E. G., C. R. DEBAY & M. P. WESTERMAN. 1960. *Biochem. Pharmacol.* **5**: 173.
16. ERDÖS, E. G., C. R. DEBAY & M. P. WESTERMAN. 1959. *Nature*. **184**: 430.
17. FUKUTO, T. R. 1957. *Advances in Pest Control Research*. **1**: 147.
18. AUGUSTINSSON, K.-B. & J. E. CASIDA. 1959. *Biochem. Pharmacol.* **3**: 60.
19. CASIDA, J. E. & K.-B. AUGUSTINSSON. 1959. *Biochim. et Biophys. Acta*. **36**: 411.
20. CASIDA, J. E., K.-B. AUGUSTINSSON & G. JONSSON. 1960. *J. Econ. Entomol.* **53**: 205.
21. AUGUSTINSSON, K.-B. & B. OLSSON. 1959. *Biochem. J.* **71**: 484.
22. AUGUSTINSSON, K.-B. & B. OLSSON. 1960. *Nature*. **187**: 4741.
23. AUGUSTINSSON, K.-B. & B. OLSSON. 1961. *Hereditas*. **47**: 1.
24. ALLISON, A. C. 1960. *Nature*. **188**: 37.
25. BERGMANN, F. & S. RIMON. 1960. *Biochem. J.* **77**: 209; cf. *idem.* 1958. *ibid.* **70**: 339.
26. AUGUSTINSSON, K.-B. & G. HEIMBÜRGER. 1954. *Acta Chem. Scand.* **8**: 753, 1533.
27. MARKERT, C. L. & F. MØLLER. 1959. *Proc. Natl. Acad. Sci. U.S.* **45**: 753.

THE ESTERASES OF MOUSE BLOOD*

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Three respects in which esterases tend to be unlike other enzymes are: they possess a low order of substrate specificity; they have been studied with a wide variety of biochemical techniques with less agreement of results than one would like; and, there are several esterases coexisting in each species, the exact number and function of each of which is still to be determined. If we are to study these esterases, techniques must be applied that will isolate the individual active protein in order that it may be characterized biochemically. The use of electrophoretic methods, by a number of investigators,^{1-7,11-18} followed by characterization of the isolated protein either on the electrophoretic column or extracted from the column, has furnished an important contribution to our understanding of this group of enzymes. The introduction of starch gel as a medium in which to carry out electrophoretic separations has effectively increased our capacity to resolve proteins. Using this technique combined with histochemical methods for dehydrogenases and esterases, Markert and Møller¹⁶ and Allen² were able to demonstrate multiple proteins that showed common properties except for differing electrophoretic mobility; these were identified by them as isozymes. Stimulated by their observations we have proceeded to study the multiple esterases previously demonstrated^{13,15} to be present in mouse blood in order to determine whether similar isozymic groups could be identified in this material.

Throughout this report we shall refer repeatedly to the esterase-active proteins located in numbered bands on the starch-gel column, demonstrated by histochemical methods. Rather than characterize and qualify this each time we have adopted the convention of referring to "bands" when, in fact, the whole concept is intended.

Materials and Methods

The mice used in this study were an inbred strain, Palid,‡ and Swiss albino. The two strains were used interchangeably since the zymogram prepared from the blood of one could not be distinguished from that prepared from the other.

Mice were anesthetized with ether, and blood was withdrawn from the left ventricle into a 21 gauge needle and a heparin (1 mg./5 ml. saline) rinsed syringe. After centrifugation at 5000 g for 10 min. the plasma was removed, the remaining red blood corpuscles (RBCs) were resuspended in 40 ml. of 0.9 per cent saline and the mixture was centrifuged. This last procedure was repeated 3 times. The RBC fraction was obtained then by discarding the supernatant. Previous studies showed no differences in zymograms prepared from serum or plasma.

The mechanics of the starch-gel electrophoresis procedure have been as pre-

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viously reported.^{12,15} The gels were poured in the morning and allowed to cool for 90 min. before using. Ten μ l. of freshly prepared samples were pipetted onto a 5- \times 13-ml. piece of filter paper for insertion at the origin. After electrophoresis for 4 hours the sliced gels were placed in the substrate diazonium mixture for a period of exactly 30 min. at 37° C. The hydrolysis of naphthol AS acetate, indoxyl acetate, or the combination of sodium taurocholate with beta naphthyl laurate was slower; these gels were incubated for 1, 12, and 2 to 3 hours respectively.

In the inhibition studies the gels were placed in the inhibitor solution 10 min. prior to the addition of the substrate mixture. Thirty-min. incubation with the inhibitor did not alter the results but caused a slight fuzzy appearance of the bands from diffusion of the enzyme in the starch. An important consideration in the inhibition studies was that when the gels were washed after staining and allowed to remain in water, the bands previously inhibited reacted with the gel-absorbed substrate and dye and then appeared less inhibited.

TABLE 1

Substrates:	α -naphthyl acetate, α -naphthyl propionate, α -naphthyl butyrate, β -naphthyl laurate, naphthol AS acetate, indoxyl acetate.
Diazonium salt:	Blue RR Salt.
Inhibitors:	eserine (physostigmine) sulfate; DFP, di-iso propylfluorophosphate; Ro 2-1250, <i>N</i> - <i>p</i> -chlorophenyl <i>N</i> -methylcarbamate of <i>m</i> -hydroxyphenyltrimethyl ammonium bromide. ⁹ Ro 2-0683, dimethyl carbamate of (2-hydroxy-5-phenyl-benzyl) trimethyl ammonium bromide; ⁸ 284C51; dibromide, 1:5-bis-(4 allyl dimethylammoniumphenyl) pentan-3-one dibromide. ¹⁰
Activator:	Sodium taurocholate.

To meet this problem the inhibitor was added to the water and the gels were photographed immediately (TABLE 1).

Plasma samples from more than 200 mice were examined; the inhibitors were studied a minimum of four times at each concentration. The molar concentrations used are listed to the right of each inhibitor in the figure.

Results

Nine bands in plasma are demonstrated consistently. We have labeled the fastest moving band No. 1 and the slowest No. 9. Occasionally a faintly-staining band between bands 5 and 6 can be observed. Bands 5 and 7 are not as prominent as the others and therefore their interaction with inhibitors is more difficult to interpret. This seems to be related to the esterase activity present and to the spreading of these proteins in the electrophoretic field, resulting in a wide weak band. If the plasma sample is stored under refrigeration without freezing or is kept at room temperature for a few hours, the area of band 2 shows no esterolytic activity. Freezing retains this activity, but to avoid possible changes fresh samples were prepared each day.

All bands are demonstrated by α -naphthyl acetate, butyrate and propionate except band 9, which is not distinct with α -naphthyl acetate; α -naphthyl acetate and propionate produce a background staining of the gel as does β -naphthyl

laurate. The inhibition studies shown in FIGURE 1 were done using α -naphthyl butyrate as substrate.

Naphthol AS acetate is hydrolyzed by bands 3, 6 and 8; there is little hy-

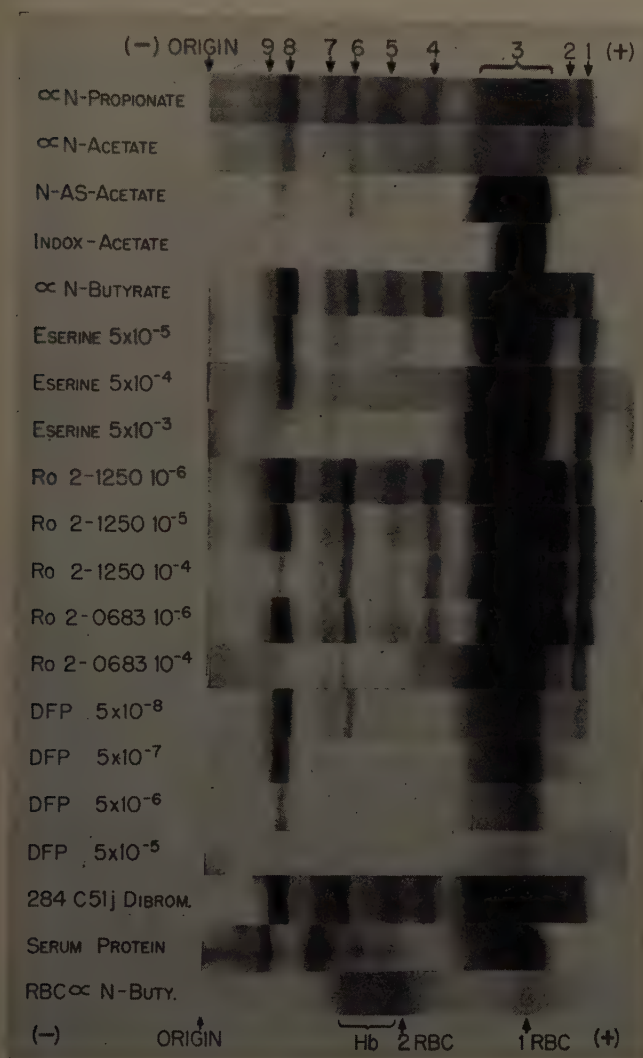


FIGURE 1.

drolysis by bands 2, 4, 5, and 7 and no hydrolysis by bands 1 and 9. Indoxyl acetate shows bands 3 and 8 and occasionally shows bands 4 and 6. Only band 0 is demonstrated by β -naphthyl laurate plus sodium taurocholate; this band is difficult to see because of intense background staining and is not included in FIGURE 1.

Eserine, in increasing concentrations, inhibits bands 2, 4, 5, 6, and 8. The relation of the inhibition of each band to the concentration of the inhibitor can be noted on the figure. Eserine has some effect on band 9 and none on bands 1, 3, and 7. Ro 2-1250 inhibits band 8 as does Ro 2-0683. Band 5 does not show a consistent pattern for reasons mentioned above. DFP inhibits bands 1, 2, 4, 5, 6, 7, and 9 at various concentrations, some bands are more sensitive and others less sensitive. At the highest concentration even bands 3 and 8 are inhibited. At the concentrations used (1×10^{-6} , 1×10^{-5} , and 1×10^{-4} molar) 284C51j dibromide is without effect.

The RBCs show 2 bands. In 2 instances a very weak band running faster than band 1 was observed. The hemoglobin is represented by a wide area (bracketed in FIGURE 1), and band 2 runs in the leading edge of this. Bands 1 and 2 are inhibited by 5×10^{-5} molar eserine and band 2 is inhibited by the weakest concentration of DFP (5×10^{-7}). Bands 1 and 2 are not inhibited by 284C51j dibromide.

Discussion

The observation that the esterase-active proteins of mouse plasma tend to hydrolyze the three short chain esters of α -naphthol with equal facility, and the contrasting zymogram which is formed when naphthol AS acetate or indoxyl acetate are used as substrates is in agreement with our previous observations concerning mouse liver and kidney.¹¹ At that time we concluded that the differences observed were due primarily to differing reaction rates on the part of enzymes in the column rather than to all or none substrate specificity. The results reported here provide no evidence for altering this conclusion, however, it is possible that the esterase of band 1, for example, hydrolyzes α -naphthol butyrate and does not hydrolyze naphthol AS acetate. It is also possible that both esterases hydrolyze both substrates, but that in the case of naphthol AS acetate a certain threshold necessary for precipitation of the dye product was not exceeded. In this way a false negative reaction would be demonstrated. The resolution of such questions must await the extraction of larger quantities of the proteins involved and their testing in systems in which the hydrolysis of the substrate alone can be evaluated.

The esterase of band 8 corresponds to the cholinesterase of plasma, the C esterase of Pintér *et al.*,¹⁸ and of Augustinsson.^{3,4} That esterases of bands 2, 4, 5, and 6 are also sensitive to eserine inhibition indicates that they may also be cholinesterases.

The esterase of band 3, the albumen associated esterase, in all probability corresponds to the A esterase of Pintér *et al.*¹⁸ and of Augustinsson.^{3,4} To identify esterases which were classified by these authors as B esterases would be difficult, although bands 1, 7, and 9 would have to be considered. In addition, another unnumbered band can sometimes be identified located midway between bands 5 and 6. This band may also be a group B or aliesterase.

The observation that the esterase of band 9 is the only enzyme demonstrated with β -naphthyl laurate and sodium taurocholate indicates that it is a lipase. An incidental observation is that this band can be well demonstrated if the columns are first incubated for one hour in acetone at 4° C. before being treated with the substrate mixture.

Perhaps our most interesting finding was in the different effects upon the esterases of mouse blood achieved by the five inhibitors used in this study. Other investigators^{1,7-10} have used these inhibitors to characterize the esterases in other species. On the basis of their results DFP, Ro 2-0683 and eserine all are effective inhibitors against cholinesterase with DFP inhibiting aliesterase as well. The inhibitors Ro 2-1250 and 284C51j are more effective against acetyl cholinesterase although higher concentrations (10^{-5} M) of Ro 2-1250 also inhibit nonspecific or pseudocholinesterase. We are particularly impressed by the selective manner in which Ro 2-0683 and Ro 2-1250 inhibit the esterase of band 8 in contrast to the multiple band inhibition of eserine. All three inhibitors clearly inhibit the principle cholinesterase of plasma, but eserine inhibits other "cholinesterases" as well. We interpret the failure of 284C51j to inhibit any of the bands to mean that acetylcholinesterase is not demonstrated by these methods. The total and unselective manner in which DFP inhibits the esterases of mouse plasma indicates that this inhibitor would be unsuited for characterizing these esterases other than that at higher concentrations it inhibits them all.

In any consideration of the esterases in plasma or serum the question of the relationship of these esterase-active proteins to the proteins in pherograms stained with amido black always arises. A comparison of the serum pherogram with the zymograms in the figure reveals that bands 3, 5, and 9 most nearly coincide with the proteins stained in the pherogram. Bands 1 and 2 would similarly coincide with pre-albumens but these proteins, although present in mouse serum, are not illustrated in FIGURE 1. The albumen-associated esterase (band 3) has been shown not to coincide in location with albumen when the proteins of mouse serum are separated by two-dimensional electrophoresis in the manner described by Smithies and Poulik.¹⁹ Whether the other esterases that seem to coincide with stainable proteins in the one dimensional zymogram would coincide if more refined methods of separation were used remains to be determined. In any event the two most prominent esterases of the zymogram, bands 3 and 8, are not simply being carried along by more abundant protein, and it is probable that most of the other esterases separated in starch-gel arrive at their location in the zymogram unattached to other proteins. The most likely counter example to this generalization is found in band 9, which closely approximates the slow alpha two ($S\alpha_2$) globulin fraction in location and in band width.

It has been suggested that the multiple bands seen in the zymogram are due to a breaking up of a lesser number of esterases during the electrophoretic procedure. To test this hypothesis the following experiment was performed. The starch-gel column was set up in the usual way but was allowed to run for only 2 hours. Bands 3 and 8 were located using a thin slab sliced from the surface of the column to insert in the substrate mixture. The remaining larger portion of these bands was then sliced from the column and inserted in a second column at the origin. These were then run in the usual way. The resulting zymogram showed only two well-separated esterase bands, corresponding to bands 3 and 8. From this we conclude that these intensely reactive bands are not contributing to the formation of the weaker bands by their own breakdown.

With respect to the two esterases obtained from washed RBCs, the fact that no inhibition was observed when 284C51j or Ro 2-1250 were used as inhibitors together with our failure to demonstrate an intensely reactive esterase in this material supposedly high in acetylcholinesterase activity, argues further against the conclusion that the esterases herein demonstrated are acetylcholinesterases. RBC esterase band 2, running in the leading edge of the hemoglobin, sensitive to eserine and DFP and better demonstrated when α -naphthyl butyrate is used as substrate than when α -naphthyl acetate is used, has a corresponding enzyme in man and in the rat.

Considering the many substances that have been used to study and characterize the esterases and the relatively small sample of these materials we have used in this investigation, it is interesting that such diversity of reaction has been observed. We conclude that it is unlikely that the esterases of mouse blood can be subdivided into groups sharing properties which would warrant their classification into isozymes.

Summary and Conclusions

The blood of mice has been analyzed for esterases using the zymogram method; 9 esterases in plasma and 2 in red blood corpuscles have been demonstrated. The effect of 6 substrates, 1 activator and 5 inhibitors upon these esterases has been determined. The results indicate that the multiple esterases present in mouse blood are not readily classifiable into subgroups containing common properties.

Acknowledgment

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References

1. ALLEN, J. M., O. ERÄNKÖ & R. L. HUNTER. 1958. A histochemical study of the esterases of the adrenal medulla of the rat. *Am. J. Anat.* **102**: 93-116.
2. ALLEN, S. L. 1960. Inherited variations in the esterases of *Tetrahymena*. *Genetics* **45**: 1051-1070.
3. AUGUSTINSSON, K. B. 1958. Electrophoretic separation and classification of blood plasma esterases. *Nature*. **181**: 1786-1789.
4. AUGUSTINSSON, K. B. 1959. Electrophoresis studies on blood plasma esterases. I. Mammalian plasmata. *Acta Chem. Scand.* **13**: 571-592.
5. DEGROUCHY, J. 1958. Répartition des activités estérasiqes et phosphatasiques du sérum humain par rapport aux séroprotéines actuellement connues. *Rev. Fr. clin. biol.* **3**: 881-884.
6. DUBBS, C. A., C. VIVONIA & J. M. HILBURN. 1960. Subfractionation of human serum enzymes. *Science*. **131**: 1529-1530.
7. GOUTIER, R. 1956. Étude électrophorétique des estérases sériques et de la fixation du $DF^{32}P$ dans le sérum, chez le lapin et le cobaye. *Biochim. Biophys. Acta.* **19**: 524-534.
8. HAWKINS, R. D. & J. M. GUNTER. 1946. Studies on Cholinesterases 5. The selective inhibition of pseudocholinesterase *in vivo*. *Biochem. J.* **40**: 192-197.
9. HAWKINS, R. D. & B. MENDEL. 1949. Studies on Cholinesterase 6. The selective inhibition of true cholinesterase *in vivo*. *Biochem. J.* **44**: 260-264.
10. HOLMSTEDT, B. 1957. A modification of the thiocholine method for the determination of cholinesterase. 1. Biochemical evaluation of selective inhibitors. *Acta Physiologica Scand.* **40**: 322-330.
11. HUNTER, R. L. & M. S. BURSTONE. 1960. The zymogram as a tool for the characterization of enzyme substrate specificity. *J. Histochem. Cytochem.* **8**: 58-62.
12. HUNTER, R. L. & C. L. MARKERT. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science*. **125**: 1294-1295.
13. HUNTER, R. L., J. M. DENUCE & D. S. STRACHAN. Serum esterases in mice, rats and

man using the two-dimensional zymogram method. 1st Intern. Congr. Histochem. Cytochem. In press.

14. LAWRENCE, S. H., P. J. MELNICK & H. E. WEIMER. 1960. A species comparison of serum proteins and enzymes by starch gel electrophoresis. *Proc. Soc. Exptl. Biol. Med.* **105**: 572-575.
15. MARKERT, C. L. & R. L. HUNTER. 1959. The distribution of esterases in mouse tissue. *J. Histochem. Cytochem.* **7**: 42-49.
16. MARKERT, C. L. & F. MØLLER. 1959. Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci.* **45**: 753-763.
17. PINTÉR, I. 1957. Esterase activity of serum protein fractions. *Acta Physiol. Hung.* **11**: 39-44.
18. PINTÉR, I., G. SÁVAY & B. A. CSILLIK. 1954. Szűrőpapírelektroforézis útján szétválasztott fehérjefrakciók enzyimaktivitásának kimutatása. *Kísér. Orvostud.* **6**: 513-515.
19. SMITHIES, O. & M. D. POULIK. 1956. Two-dimensional electrophoresis of serum proteins. *Nature.* **177**: 1033.

SEPARATION AND CHARACTERIZATION OF LACTIC DEHYDROGENASES FROM SERUM AND FORMED ELEMENTS OF HUMAN BLOOD*

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Electrophoretic separation of serum proteins has provided a tool for a more critical examination of serum lactic dehydrogenase (LDH) than is obtainable in a single serum assay. The differences between electrophoretic LDH patterns obtained from sera in health and in certain diseases¹⁻⁴ cannot be completely understood without a thorough knowledge of the constituents of the patterns. To study the properties of these constituents thoroughly it is necessary to obtain them in pure form.

This report describes the development of procedures for the separation and purification of active LDH fractions. Some properties of partially purified fractions are also noted.

Materials and Methods

Extract of formed elements. Whole blood, 2 weeks to 2 months old, was obtained from the blood bank and allowed to stand at 4° C. until the formed elements had settled. The plasma was removed by aspiration, and the formed elements were lysed by the addition of 3 volumes of water. After freezing and thawing the mixture was dialyzed for several hours against distilled water. The precipitate that formed was removed by centrifugation, and the supernatant solution was employed as the active extract for the investigation of purification procedures.

Adsorbents. Aluminum hydroxide, U.S.P., dried gel, medium powder† was used, also carboxymethyl-cellulose and diethylaminoethyl-cellulose.‡ Calcium phosphate gel, prepared by the method of Keilin and Hartree,⁵ was boiled for 10 min. and cooled slowly. The gel suspension used in these experiments contained approximately 250 mg./ml.

Calcium phosphate gel column. A cotton plug was packed into the bottom of a column 1 to 2 cm. in diameter, and a few milliliters of gel suspension was added. After this had settled, the gel suspension containing LDH was layered over it to give a column of about 10 cm. in length. The flow rate could be increased, especially when vacuum was not used, by the addition of 0.5 gm cellulose powder (Whatman ashless, standard grade) per 10 ml. of original gel suspension.

LDH assay. LDH activity was determined by the method described by Hill.⁴ Specific activity was expressed μM DPNH oxidized per minute per milligram protein.

Protein assay. Protein concentrations were determined by the method of

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† Obtained from J. T. Baker Co., Phillipsburg, N.J.

‡ Bio-Rad Laboratories, Richmond, Calif.

Lowry *et al.*⁶ The method was calibrated with crystalline bovine serum albumin (Armour & Co.) and checked against optical density measurements at 260 and 280 $m\mu$.

LDH fractionations. Electrophoretic separations of LDH components were conducted at room temperature in the Spinco Model CP continuous-flow paper

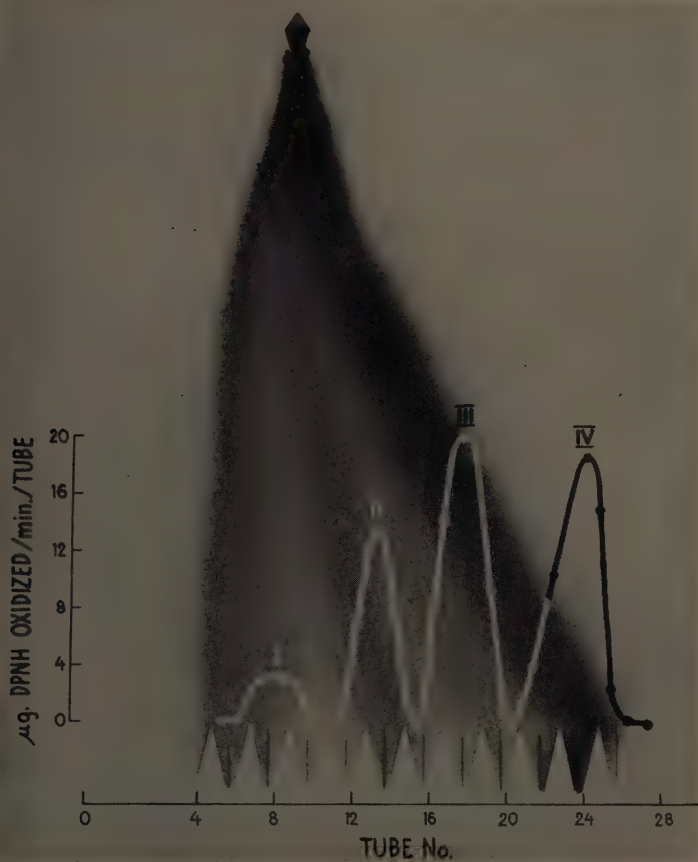


FIGURE 1. Electrophoretic profile of LDH activity from the serum of an apparently healthy individual, superimposed on the dyed sheet which shows protein distribution. See text.

electrophoresis unit in the presence of 0.02 ionic strength barbital buffer, pH 8.6. A current of 30 mAmp. at 450 v was employed.

Results

FIGURE 1 shows the electrophoretic profile of LDH activity from the serum of an apparently healthy individual superimposed on the dyed sheet that shows protein distribution. Fractions I, II, and III appear with the γ -globulins, β -globulins, and α_2 -globulins respectively, and Fraction IV with the α_1 -globulin-albumin fraction.

FIGURE 2 compares the electrophoretic pattern of the formed element extract with the pattern for serum shown in FIGURE 1. On the basis of electrophoretic mobilities, the several LDH fractions found in such extracts are indistinguishable from the corresponding fractions found in serum. Aging of blood caused a decrease in Fraction II activity and, in many samples, Fraction I was entirely absent. However, Fraction II was recovered in sufficient quantity for the study of purification procedures.

A simple aqueous extract of 1 U. (480 ml.) of blood required more than 2 weeks to fractionate in 2 electrophoresis instruments. Fraction II was highly

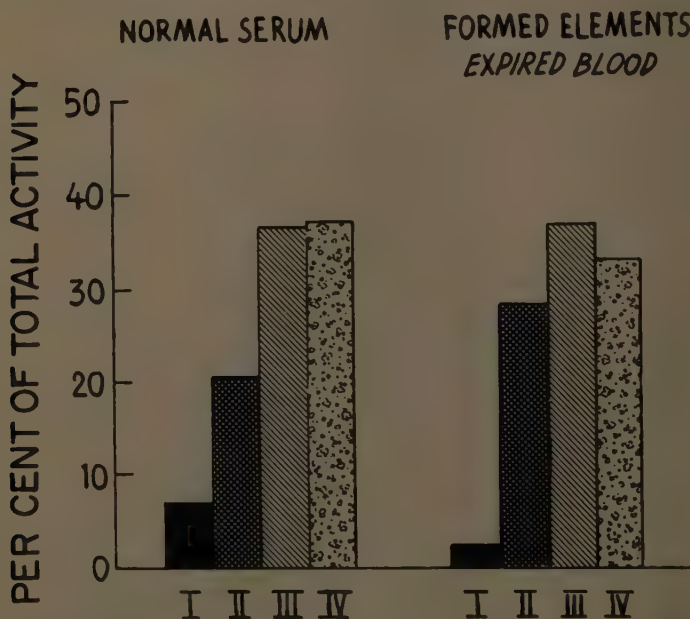


FIGURE 2. Comparison of electrophoretic LDH patterns from serum and an extract of the formed elements of blood. The serum pattern was that of the serum shown in FIGURE 1. The formed elements were separated from blood several weeks old. See text.

colored with hemoglobin, Fraction III had a red coloration, and the LDH activities of the fractions were lowered by dilution.

Methods were explored for the removal of hemoglobin and concentration of the extract prior to electrophoresis. Three alternative procedures have been devised: the aluminum hydroxide method, the calcium phosphate gel method, and the carboxymethyl-cellulose method.

Aluminum-hydroxide method. In this method 70 gm. of aluminum hydroxide gel was stirred for 10 min. with the extract from 1 U. of blood. Additional gel was added, if necessary, for complete adsorption of LDH activity. The quantity of adsorbent required varied with different batches of the gel. The mixture was centrifuged and the dark red supernatant solution discarded. The gel was washed with water several times to remove most of the hemoglobin, and the washes were discarded. The LDH activity and some

residual color were removed by four extractions, each with 100 to 150 ml. of 0.1 *M* disodium hydrogen phosphate solution. The yield of LDH was only slightly increased by a fifth extraction. The combined extracts were concentrated to about one-fifth volume by dialysis against a 30 per cent solution of polyvinylpyrrolidone (PVP) in 0.02 ionic strength barbital buffer, pH 8.6. There was little or no loss in activity during the concentration procedure. The gel-treated concentrate from 1 U. of blood required only 2 days for electrophoretic separation, and the fractions were high in LDH activity with relatively little color.

Calcium-phosphate-gel method. This gel could replace aluminum hydroxide in the above procedure. Some difficulty, however, was encountered in the preparation of uniform batches of gel. The physical properties of the calcium

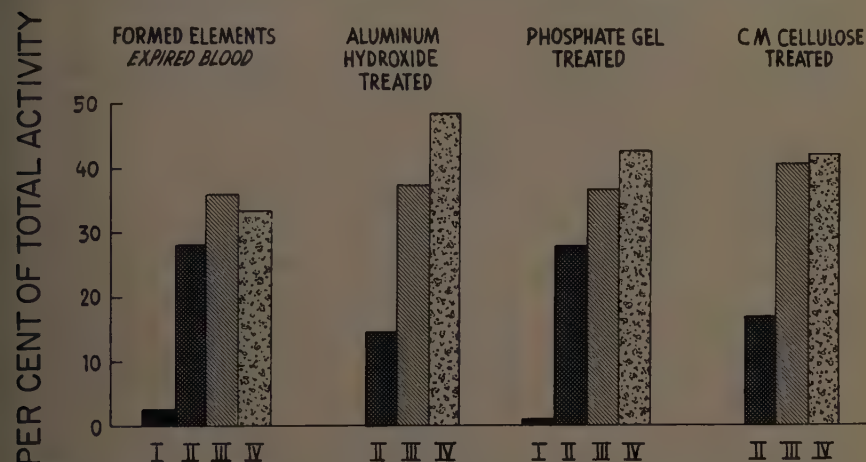


FIGURE 3. LDH patterns of formed element extracts after treatment to remove hemoglobin. See text.

phosphate gel were less desirable, and more hemoglobin was adsorbed on the gel, which could not be removed by water.

Carboxymethyl-cellulose method. Under certain conditions carboxymethyl (CM)-cellulose adsorbed hemoglobin, but not LDH activity. A paste was prepared from 15 gm. CM-cellulose and 100 ml. of water. To this was added 100 ml. of extract, and the mixture was stirred for 10 min. The CM-cellulose, containing the hemoglobin, was removed by centrifugation and washed once with 50 ml. of water. Recovery of LDH could be increased slightly by an additional wash. Very little hemoglobin color was evident in the active supernatant solution. This solution was concentrated by dialysis against PVP.

A recovery of 75 to 80 per cent of the LDH activity and a four- to sixfold increase in specific activity were obtained by each of the three methods.

The dialyzed concentrates were fractionated electrophoretically. LDH patterns, as compared to the pattern from the original extract, are shown in FIGURE 3. The respective electrophoretic mobilities for the three fractions after treatment were the same as for those in untreated extract. Relative mobilities of

the LDH fractions were also determined by starch block electrophoresis, both before and after separation by continuous flow electrophoresis. Results of these two electrophoretic methods were found to be in good agreement. In extracts treated with aluminum hydroxide or phosphate gel, Fraction II contained considerable color from hemoglobin. This fraction from CM-cellulose treated extract contained little color. Fraction III from all the extracts was yellow. Fraction IV from extracts treated with aluminum hydroxide and CM-cellulose was highly colored and brownish. There was very little of this color in the phosphate gel treated extract and none in the untreated extract. The brown color suggests the presence of heme that combines with a protein appearing in Fraction IV. The specific activities of the fractions were not significantly increased by electrophoresis. In a few cases the specific activity of Fractions II was decreased slightly and, of Fraction III, increased slightly.

After electrophoretic separation the fractions varied greatly in stability. TABLE 1 shows the loss in activity during storage in barbital buffer at 4° C. Fractions III and IV are much more stable than II. A previous report² described an increase in heat stability by the addition of albumin or reduced

TABLE 1
STABILITY OF DILUTE FRACTIONS AT 4° C.

Fraction	Per cent of original activity		
	7 Days	21 Days	36 Days
I	0	0	0
II	55	40	25
III	89	68	48
IV	100	100	100

glutathione. However, the addition of these substances to Fractions II and III had no effect on stability at 4° C. Freezing of the formed elements or of a crude aqueous extract did not significantly destroy LDH activity. The treated extracts and the separated fractions lost considerable activity upon freezing and thawing. Treated extracts and fractions have been stored, without appreciable loss of activity, for several months at 4° C., after the addition of ammonium sulfate to give an 80 per cent saturated solution. This appears to be the best method for storing and accumulating fractions for subsequent purification steps, although Fraction II has shown a gradual decline in activity under these conditions.

Each fraction was further purified on a column of calcium phosphate gel. Gel was added to the dialyzed fraction until the supernatant solution contained no activity. After centrifugation the gel was washed twice with water, suspended in water, and introduced into a gel column. Elution of LDH activity from the column was effected by the successive addition of 0.01, 0.02, and 0.1 *M* solutions of disodium hydrogen phosphate.

TABLE 2 shows the results of column elutions. Recovery of activity from each of these columns was quantitative.

When Fraction II was eluted, most of the color appeared with the 0.01 and

0.02 *M* eluates, but no activity appeared until 0.1 *M* phosphate was employed. The activity was recovered in a small volume of eluate, which contained some color.

When Fraction III was eluted, some activity and most of the color appeared with the 0.01 and 0.02 *M* eluates. However, about 80 per cent of the activity appeared in a relatively small volume of the 0.1 *M* eluate, with very little color.

The behavior of Fraction IV was similar to that of III except that more activity was eluted with the 0.01 *M* phosphate. Approximately 50 per cent of the activity appeared in this fraction and 50 per cent in the 0.1 *M* eluate, with more color in the latter.

Elutions for all columns were regulated according to the appearance of color and activity in the eluate. The molarity of the phosphate solution was not increased until LDH activity was decreasing or the eluate was nearly colorless. Little work has been done to date comparing the 0.01 and 0.1 *M* eluates from III and IV, but further experiments are now in progress. The possibility cannot be ignored that they are identical but associated with different proteins.

TABLE 2
ELUTION OF LDH FROM CALCIUM PHOSPHATE GEL COLUMNS

Na ₂ HPO ₄ solution	Fraction					
	II		III		IV	
	Per cent activity	Vol. (ml.)	Per cent activity	Vol. (ml.)	Per cent activity	Vol. (ml.)
0.01 <i>M</i>	0	60	3	18	48	35
0.02 <i>M</i>	0		18	30	6	56
0.10 <i>M</i>	100	6	79	8	46	15

In one series of experiments diethylaminoethyl (DEAE)-cellulose gave a clearer separation of the 0.01 and 0.1 *M* eluates from Fractions III and IV. In both cases the 0.01 *M* eluates contained activity, but no LDH activity was eluted with 0.02 *M*. A second fraction appeared with the 0.1 *M* phosphate. The use of this adsorbent is now under investigation.

The active eluate of II and the active eluates of III and of IV were precipitated by 70 per cent saturation with ammonium sulfate. The precipitates were washed twice with 70 per cent saturated ammonium sulfate, and the LDH activity extracted from the precipitate by a 55 per cent saturated solution. The specific activities of the 55 per cent extracts were 115 to 120 times that of the original extract of the formed elements, and some of these extracts have been practically colorless. Electrophoretic mobilities were comparable to those of fractions present in the original extract. These active fractions were quite stable when stored in a solution of 55 per cent saturated ammonium sulfate at 4° C. After storage for 2 mo. under these conditions, Fraction II retained about one half, and Fractions III and IV about three fourths of their original activity. Storage for 1 mo. in water resulted in the loss of all but about 10 per cent of the original activity of all fractions. Activity is rapidly lost in acidic solutions.

Michaelis-Menten (K_m) constants for pyruvate for the three purified fractions did not differ significantly over a pH range of 7.0 to 8.0 (TABLE 3). K_m for all fractions is somewhat smaller at the lower pH values, as shown previously, for fractions obtained from serum.²

Fraction I, with the mobility of γ -globulin (FIGURE 1), has been low or absent

TABLE 3
MICHAELIS-MENTEN CONSTANTS (K_M) FOR PYRUVATE

Fraction	K_m (pyruvate) $\times 10^4$ M/l.					
	pH 7.0	pH 7.2	pH 7.4	pH 7.6	pH 7.8	pH 8.0
II	1.4	1.5	1.5	2.0	4.2	4.8
III	1.1	1.5	1.5	2.5	3.3	4.1
IV	1.0	1.2	1.6	1.8	3.8	4.1

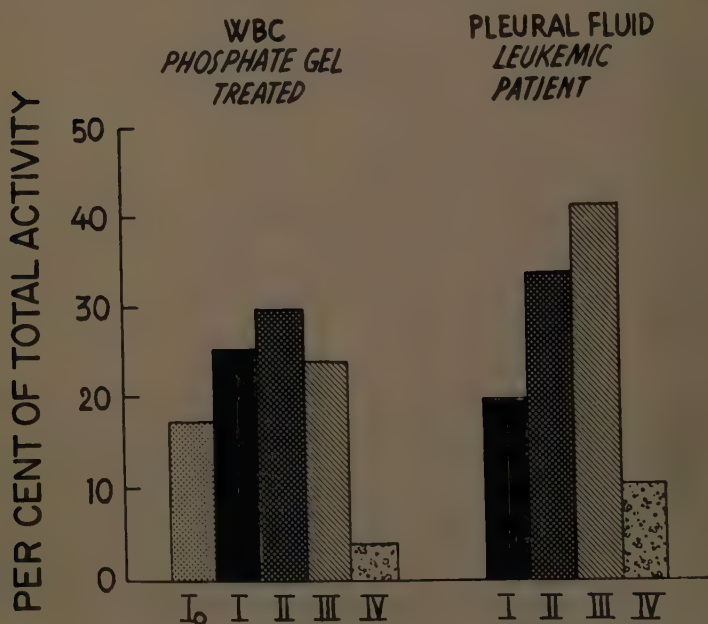


FIGURE 4. LDH patterns of leukocytes (WBC) and pleural fluid from a leukemic patient. See text.

in the formed element extracts utilized in these investigations. However experiments have indicated that leukocytes and platelets have sufficient Fraction I for studies of purification methods (FIGURE 4). The pattern for leukocytes was obtained from cells that had been frozen for about one year. Frozen platelets gave approximately the same pattern. In both cases, five active fractions were observed upon electrophoresis, and the additional fraction designated I₀, had a lower electrophoretic mobility than Fraction I. Fresh

leukocytes and platelets will be used for studies of Fraction I and, possibly, I_0 . Another good and plentiful source of these fractions was pleural fluid from a patient with leukemia and a very high serum LDH.

The samples of serum investigated to date have been treated differently from the formed elements. One and one-half volumes of an 80 per cent saturated solution of ammonium sulfate were added to the serum. The precipitate, containing very little LDH activity, was removed by centrifugation. The supernatant solution was dialyzed against distilled water to remove sulfate. After electrophoresis the fractions were purified on calcium phosphate gel columns. Because of the scarcity of highly active sera, details of this procedure have not yet been completely worked out.

Discussion

Serum or plasma from healthy individuals is too low in LDH activity to be used in preparing the large quantities of active fractions needed for development of purification procedures. As large volumes of high activity sera from selected leukemia and cancer patients are almost impossible to obtain, it was necessary to locate some other very active human material that would be easily available in large quantities.

Previous investigations² have shown that the formed elements of blood are high in LDH activity, and that electrophoresis yields LDH fractions with mobilities similar to the mobilities of serum fractions. Expired whole blood from the blood bank was available in large quantities, and it proved to be a satisfactory source of certain LDH fractions that could be used for the study of various purification procedures.

Once purification procedures have been standardized, extracts of leukocytes and platelets will be the preferred sources for four, and possibly five, fractions with LDH activity. Although whole blood is more readily available, the viscosity and dark color of the extracts that it yields impose limitations on the efficiency of purification procedures. The steps required to remove large quantities of hemoglobin entail serious losses of the more labile fractions of LDH activity.

Summary

An investigation has been made of human source materials for the preparation of fractions with LDH activity. Purification procedures have been developed that give approximately 115- to 120-fold purification of 3 fractions from the formed elements of blood. Future plans include preparation of greater quantities of these purified fractions and investigation of procedures for further purification. Further characterization of the purified fractions will be attempted.

References

1. SAYRE, F. W. & B. R. HILL. 1957. Fractionation of serum lactic dehydrogenase by salt concentration gradient elution and paper electrophoresis. *Proc. Soc. Exptl. Biol. Med.* **96**: 695-697.
2. HILL, B. R. 1958. Further studies of the fractionation of lactic dehydrogenase of blood. *Ann. N. Y. Acad. Sci.* **75**(1): 304-310.

3. HILL, B. R. 1959. Electrophoretic fractionation of serum lactic dehydrogenase. *Proc. Am. Assoc. Cancer Research.* **3**: 27.
4. HILL, B. R. 1961. Electrophoretic fractionation of serum lactic dehydrogenase. *Cancer Research.* **21**: 271-274.
5. KEILIN, D. & E. F. HARTREE. 1938. On the mechanism of the decomposition of hydrogen peroxide by catalase. *Proc. Roy. Soc. London.* **124B**: 397-405.
6. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL. 1951. Protein measurement with the phenol reagent. *J. Biol. Chem.* **193**: 265-275.

SIGNIFICANCE OF THE HETEROGENEITY OF LACTIC DEHYDROGENASE ACTIVITY IN HUMAN TISSUES*

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The heterogeneity of lactic dehydrogenase (LDH) activity in human blood has become generally accepted.¹⁻⁸ Through the development of the concept of "isozymes" by Markert and Møller, who proposed the term to refer to electrophoretically distinguishable enzymes with similar substrate specificities, the presence of more than one form of an enzyme within a cell has been elevated to a general biological principle.⁸ These workers demonstrated that the cells of some embryological tissues differ from the adult cells in their complement of LDH isozymes.⁸ Kaplan *et al.* placed the phenomenon of the heterogeneity of enzymes in another biological setting by comparing lactic dehydrogenases from different species, and by suggesting a phylogenetic scheme of the evolution of enzymes from lower to higher forms.⁹

The clinical significance of LDH heterogeneity was suggested by the initial observation of multiple LDHs in human serum and red blood cells when it was shown that sera from patients with myocardial infarction had an elevation of the electrophoretically fastest migrating isozyme, whereas sera from patients with leukemia revealed a selective elevation of the second-fastest migrating isozyme.¹ These results have been confirmed by recent intensive studies of Wróblewski and Gregory,¹⁰ Wieme,¹¹ Hess and Walter,¹² and Hill.¹³ The present paper extends the clinical applications of LDH isozymes in serum in disease states, and lends support to the hypothesis that elevation of individual LDH isozymes in sera results from the release of enzymes into blood from damaged tissues. The physicochemical properties of the five LDH isozymes in human tissues were investigated, and evidence is presented showing that, in addition to their differences in electrophoretic mobility, the LDH isozymes vary in their kinetic behavior.

Materials and Methods

Materials. Four gm. each of heart, kidney, liver, lung, pancreas, and skeletal muscle were obtained within 12 hours after death of patients from diseases not affecting the metabolism of the tissues under study. The tissues were washed thoroughly in cold normal saline to remove the majority of red cells and were rinsed in cold water to lyse any remaining erythrocytes. However, even after careful washing, certain tissues such as the liver, lung, and kidney revealed residual hemoglobin in the homogenates. Homogenates were prepared in 10 ml. of barbital buffer, pH 8.6, ionic strength 0.1, using a mortar and pestle and then submitting the tissues to further grinding in a Potter-Elvehjem motor-driven tissue homogenizer. Centrifugation of these extracts for 40 min. at 15,000 g at 4° C. produced a supernatant fluid that was decanted and diluted with an equal volume of barbital buffer, pH 8.6.

In several experiments a hemolysate prepared from human red cells¹⁴ was

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fractionated by starch-block electrophoresis; the eluates were assayed for LDH activity; and in some cases the material from the peak tubes was applied to starch gels and rerun by starch-gel electrophoresis.

Human white cells were obtained from 40 ml. of heparinized blood by the method described by Hirsch and Church.¹⁵ The LDH activity was extracted from this purified preparation of leukocytes by freezing in acetone and dry ice, followed by thawing. The white blood cell extract was separated electrophoretically, and the eluates were assayed for LDH activity.

Serum from a patient with hepatitis, from a patient with chronic granulocytic leukemia, from a patient with myocardial infarction, and from a healthy control was separated simultaneously on a starch block. Sera from patients with acute pancreatitis were also separated electrophoretically. Hemorrhagic shock was produced in healthy mongrel dogs (average weight 20 kg.) by the elevated reservoir technique.^{16,17} The electrophoretically separated sera were examined for LDH activity before and after hemorrhagic hypotension.

Electrophoresis. Four ml. of the diluted supernatant was separated electrophoretically on a starch-supporting medium in barbital buffer, pH 8.6, ionic strength of 0.1.¹⁸ After electrophoresis at 4° C. for 36 hours at 400 v and 1200 mAmp., the starch block was cut into 0.5 inch segments, the protein eluted with 5.0 ml. barbital buffer, and its concentration determined.¹⁸ Between 85 and 98 per cent of the LDH activity was recovered from the block following electrophoresis. Electrophoresis of these tissue homogenates was also performed on starch gels according to the method of Smithies,¹⁹ employing the discontinuous buffer system of Poulik.²⁰ A gradient of 6 v per centimeter was maintained for 12 to 16 hours.

LDH assay. LDH activity of eluates from the starch block was determined spectrophotometrically according to the method devised by Kubowitz and Ott,²¹ in which the decrease in optical density at 340 m μ is measured as the reduced diphosphopyridine nucleotide (DPNH) is oxidized to diphosphopyridine nucleotide (DPN). Each eluate was incubated for 20 min. at room temperature with 0.2 ml. of 0.003 *M* DPNH and 2.5 ml. of barbital buffer, pH 8.6, ionic strength 0.1. The mixture was transferred to a Beckman cuvette of 1 cm. path length. Sodium pyruvate (0.1 ml. of 0.001 *M*) was added, and the decrease in absorption at 340 m μ was measured in the Beckman DU spectrophotometer. Readings were obtained every 30 sec. for 3 min. One U. of dehydrogenase activity was defined as a decrease in the optical density at 340 m μ of 0.001 per minute per milliliter of eluate.

LDH activity was identified histochemically directly on the starch gel by the use of nitro-blue tetrazolium, according to a recently revised technique of Markert⁸ (C. L. Markert and E. Appella, personal communication). Phenazine methosulfate served as the electron transporter. A brilliant purple color produced by the reduction of the tetrazolium salt to the formazan appeared at the site of LDH activity on the starch gel. The gel is overlaid directly with the following solution and incubated at 37° C. for 1 hour in the dark: DL lactate 0.5 *M* (12.0 ml.), DPN 10 mg./ml. (2.4 ml.), nitro BT 1 mg./ml. (6.4 ml.) and Tris buffer pH 8.3 (53.0 ml.). Phenazine methosulfate 1.6 mg./ml. (7.2 ml.) is added to the above solution in the dark because of its instability in light. This method has recently been described by Dewey and Conklin.²²

Studies of LDH isozymes. The *pH* optimum for each peak of LDH activity was determined. An aliquot from the tube containing the maximal LDH activity in each peak was removed and added to barbital buffers, varying in *pH* from 7.4 to 9.2, of 0.1 ionic strength. LDH activity was then reassayed as described above.

The Michaelis-Menten constants (K_m) of lactic dehydrogenase were determined for each of the 5 peaks of LDH activity. One-thousandfold variations in the final concentration of sodium lactate (1.6×10^{-3} moles to 1.6 moles, *pH* 7.0, ionic strength 0.1) were employed. DPN was used (0.3 ml. of a 0.05 *M* solution adjusted to *pH* 7.5).²³ Barbital buffer, *pH* 8.6, ionic strength 0.1, was added to an aliquot from the tubes with peak LDH activities. The final *pH* was 8.6 in a constant volume of 3.0 ml. The mixture was assayed for LDH activity and the K_m calculated according to the method of Lineweaver and Burk.²⁴

The activity of the LDH isozymes from human tissues was studied with analogs of DPN.⁹ The deamino and the acetylpyridine analogs* were employed (0.3 ml. of a 0.05 *M* solution for each assay).

Results

Normal human serum separated by starch-block electrophoresis revealed 4 main peaks of LDH activity as seen in FIGURE 1. Most of the activity was located in peak 4; peak 5 contained the next highest activity; peak 3 revealed less activity than peak 5; and peak 2 contained least activity. This distribution is characteristic of normal human serum and has been obtained consistently.¹⁻⁴ Peak 2 is located in the γ globulin; peak 3 in the β globulin; peak 4 in the α_2 globulin, and peak 5 is found between the α_1 globulin and the albumin, as indicated by the protein curve in FIGURE 1 for leukemic serum. The proportion of the total LDH activity in each peak is altered in various disease states. FIGURE 1 shows that in serum from a patient with myocardial infarction peak 5 contained most LDH activity whereas, in leukemic serum, peak 4 became larger than in normal serum so that the ratio of LDH activity in peak 4 to that in peak 5 increased.¹ In serum from a patient with hepatitis most of the LDH activity is found in peaks 1 and 2, whereas in serum from a patient with hemorrhagic pancreatitis there appears to be an elevation of peak 3 (FIGURES 1 and 2). FIGURE 3 shows that after a period of hemorrhagic hypotension in dogs all 4 peaks of serum LDH activity are elevated; therefore it should be emphasized that when shock supervenes in myocardial infarction, leukemia, hepatitis, or hemorrhagic pancreatitis the serum LDH isozyme pattern may differ from the pattern observed when only one organ is affected. Furthermore, alterations in the serum LDH isozyme pattern will occur when hemolysis plays a prominent role in the disease process, as in pulmonary infarction, hemorrhagic pancreatitis, and some cases of shock.

The LDH isozymes of various human tissues are seen in FIGURES 4 and 5. Five isozymes were observed, although not all 5 appeared in each of the tissues. Liver exhibited 1 major and 4 minor ones, and lung exhibited 2 major peaks and 3 minor ones. Kidney, pancreas, red cells, and white cells revealed 4

* Obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio.

peaks, and heart and skeletal muscle had 3. The percentage of the total LDH activity found in each peak varied within the limits indicated in TABLE 1, and it will be seen that the activities of peaks 3 to 5 in tissues such as liver and lung, from which red blood cells could not be completely removed, had large standard deviations probably due to the varying contributions from red

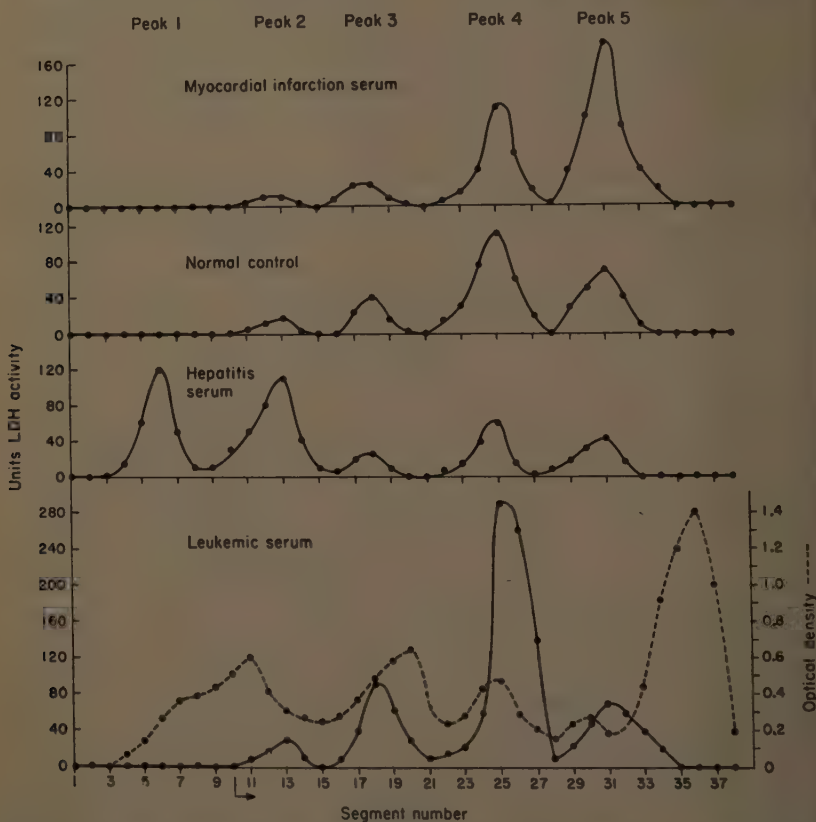


FIGURE 1. Distribution of lactic dehydrogenase activity in normal human serum and sera from patients with myocardial infarction, chronic granulocytic leukemia, and infectious hepatitis. These four sera were separated simultaneously on a starch block. Note that each patient shows alterations in individual LDH activity peaks from the normal pattern. The curve with the broken line for the leukemic serum represents the electrophoretic pattern. The origin is indicated by the arrow.

blood cells of LDH isozymes 3 to 5. Furthermore each tissue had a characteristic distribution of LDH activity. Most of the LDH activity in heart appeared in peak 5, with a small component in peak 4 whereas, in liver and skeletal muscle, the majority of the activity was found in peak 1. In homogenate of pancreas, peaks 3 and 5 contained most of the LDH activity (FIGURE 2) whereas, in lung, most of the activity was in peaks 3 and 4, with appreciable activity in peaks 1, 2, and 5. In kidney homogenate most of the LDH activity was divided between peaks 4 and 5, and in leukocyte extract peak 4

was highest (TABLE 1). The mobility of each of the activity peaks in 1 tissue resembled the mobility of the corresponding peaks in other tissues, as shown in FIGURES 4 and 5.

The pH optima of the 5 LDH isozymes were compared. TABLE 2 shows that the values were quite similar, ranging from 7.8 to 8.5. These differences in pH optima were not statistically significant.

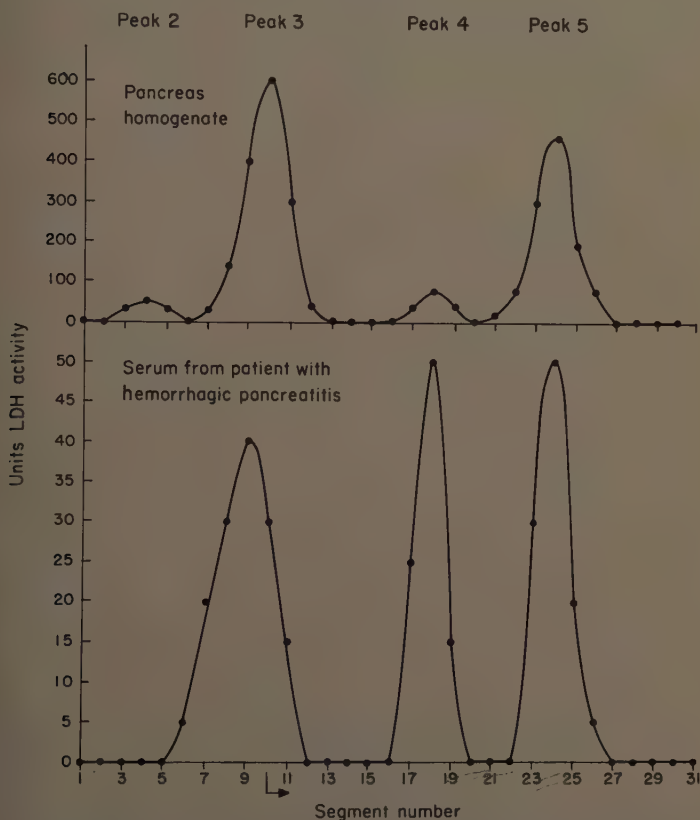


FIGURE 2. The upper curve represents the lactic dehydrogenase activity of homogenate of human pancreas separated simultaneously on a starch block with serum from a patient with acute pancreatitis (*lower curve*). Note the elevation of serum peak 3 that contained 42 per cent of the total LDH activity. Normally this peak contains 19 per cent.¹ It corresponds in electrophoretic mobility to the peak in pancreatic tissue with highest LDH activity.

The Michaelis-Menten constants (K_m) were calculated for the 5 activity peaks. The values obtained (TABLE 3) revealed no significant differences among peaks 3, 4, and 5. However, the K_m of peaks 1 and 2 (4.2 and 5.6×10^{-5} moles respectively) were greater than the K_m of peaks 3, 4, and 5 (1.0 to 2.0×10^{-5} moles).

Differences were observed in the kinetic behavior of the LDH isozymes toward 2 DPN analogs, as shown in TABLE 4. The ratio of maximum LDH activity between the reaction with deamino-DPN as coenzyme and the re-

action with acetylpyridine-DPN as coenzyme was 14 for isozymes 4 and 5, whereas it was 2 for isozyme 1. Standard deviations from the mean are included in TABLE 4.

The activity peaks isolated from the starch block were rerun on a second starch block. No alterations in mobility from the starting material were observed; nor did a single peak yield more than a single peak when rerun. FIGURE 6 shows peak 1 from liver homogenate rerun on a second starch block under identical conditions, and the rerun on a second starch block of a pool of peak 5 from heart homogenate and peak 4 from kidney homogenate.

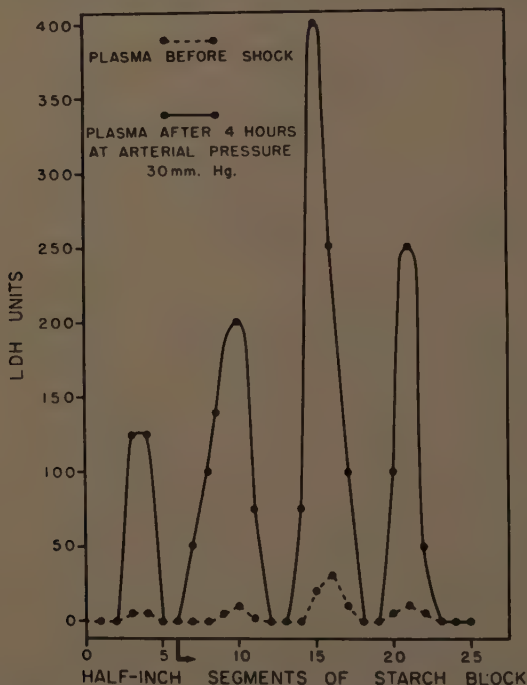


FIGURE 3. Distribution of lactic dehydrogenase activity in normal dog plasma (dotted line) and in plasma from the same dog after 4 hours at arterial pressure 30 mm. Hg (solid line). Both plasmas were separated simultaneously on a starch block. Reproduced by permission of the Society for Experimental Biology and Medicine.

The tissue homogenates when separated by starch-gel electrophoresis yielded patterns similar to those observed on a starch block. FIGURE 7 shows the 3 main LDH isozymes in human hemolysate separated by starch-gel electrophoresis with Na_2HPO_4 -citric acid buffer, pH 7.0. The 3 isozymes of hemolysate isolated from a starch block and rerun on the gel are also shown to demonstrate that no change in mobility has occurred under the conditions employed.

Discussion

Utilizing starch-block electrophoresis, 3 main peaks of LDH activity in human serum and red blood cells were demonstrated,¹ with a small fourth

peak present in the gamma-globulin region.² It was shown that in normal serum approximately 47 per cent of the total LDH activity was present in the α_2 peak, 34 per cent between the α_1 globulin and albumin, and 19 per cent in the β globulin.¹ In cases of myocardial infarction the α_1 peak contained more than the normal portion of the total LDH activity, and in leukemic serum the α_2 peak became differentially elevated.¹ It was suggested that in disease states alterations from the normal pattern arose as a result of release of enzyme from the damaged tissue. This suggestion received support

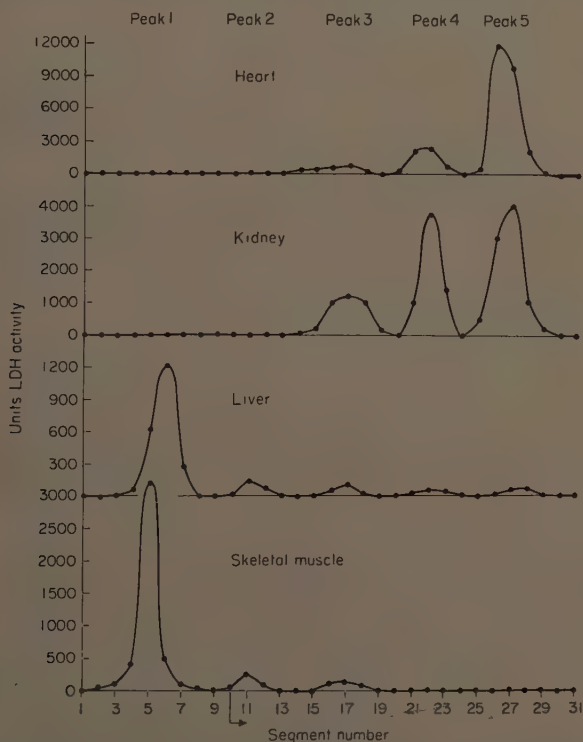


FIGURE 4. Distribution of lactic dehydrogenase activity in homogenates of human heart, kidney, liver, and skeletal muscle. These tissue homogenates were separated simultaneously on the same starch block. The origin is indicated by the arrow.

from electrophoretic studies of sera of dogs in hemorrhagic shock.¹⁷ In hemorrhagic shock widespread tissue damage results from generalized anoxia, and it was found that all 4 peaks of LDH activity were elevated in shock serum.¹⁷ Furthermore, the data presented in this paper on LDH isozymes in human tissues lends support to the idea of enzyme release from damaged tissues as a cause of alterations in the pattern of LDH isozymes in serum. The selective elevation of the LDH isozyme with the mobility of α_1 globulin in sera of patients with myocardial infarction correlates with the observation that these are the locations of most of the LDH activity in heart homogenate.^{1-3,8,25} The elevation of the peak of LDH activity in the α_2 globulin in certain leukemic sera¹ correlates with the observation that the majority of LDH activity

in leukocytes is contained in this activity peak. The elevation of the LDH activity peak in the β globulin and the appearance of a large activity peak in the γ globulin in serum from some patients with hepatitis are in harmony with the observation that the majority of the LDH activity in liver homogenate re-

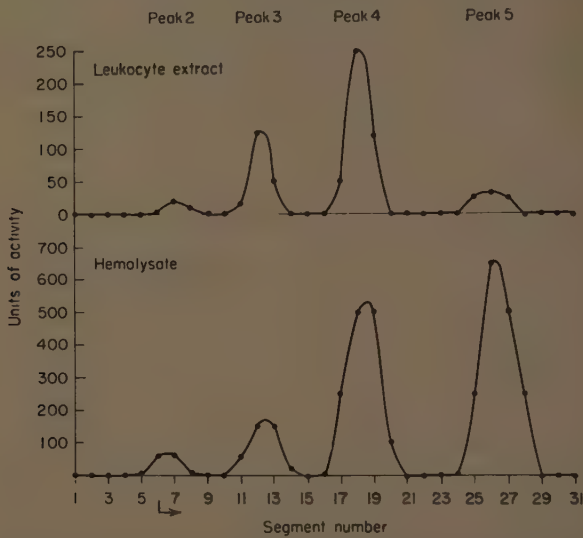


FIGURE 5. Distribution of lactic dehydrogenase activity in normal human hemolysate and leukocyte extract. Reproduced by permission of *The Journal of Clinical Investigation*

TABLE 1
LACTIC DEHYDROGENASE ACTIVITY IN FRACTIONS OF HUMAN TISSUES WITH
STANDARD DEVIATIONS

Tissue	No. of cases	Per cent total activity in each peak				
		1	2	3	4	5
Heart	10			3 ± 2.7	24 ± 4.6	73 ± 5.0
Kidney	10		1 ± 2.7	12 ± 4.0	44 ± 5.7	43 ± 6.6
Liver	10	41 ± 10.4	23 ± 11.1	25 ± 8.0	8 ± 7.2	3 ± 8.2
Skeletal muscle	6	79 ± 8.2	16 ± 8.1	5 ± 4.0		
Hemolysate	5		1 ± 1.8	12 ± 2.9	44 ± 4.3	43 ± 2.2
White blood cells	5		6 ± 3.6	33 ± 6.1	49 ± 5.1	12 ± 3.6
Lung	3	12 ± 0.6	5 ± 2.9	35 ± 16.8	34 ± 12.0	14 ± 12.3
Pancreas	3		2 ± 0.6	49 ± 1.7	12 ± 5.2	37 ± 2.9

TABLE 2
pH OPTIMA OF 5 PEAKS OF LDH ACTIVITY

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Heart			8.0	8.1	8.2
Hemolysate			8.0	8.2	8.5
Kidney			7.8	8.1	8.3
Liver	8.3	8.3			
Skeletal muscle	8.2	8.2			

sides in peaks with similar mobilities. Furthermore the elevation of the β -globulin peak of LDH activity in serum from patients with hemorrhagic pancreatitis correlates with the observation that the majority of the LDH activity in homogenate of pancreas has similar electrophoretic mobilities. In one pa-

TABLE 3
MICHAELIS-MENTEN CONSTANTS (LACTATE) $\times 10^{-4}$ M/l. FOR 5 PEAKS OF LDH ACTIVITY

	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Heart			0.19	0.15	0.12
Kidney			0.10	0.20	0.10
Liver	0.22	0.15			
Skeletal muscle	0.26	0.24			

TABLE 4
RATIO OF LDH ACTIVITY WITH DPN ANALOGUES AS COENZYME

Peak	Tissue	Ratio of LDH activity deamino-DPN/3-acetylpyridine- DPN
1	Liver	3 ± 1
1	Skeletal muscle	3 ± 1
4	Heart	14 ± 2
4	Kidney	14 ± 2
5	Heart	14 ± 2
5	Kidney	14 ± 2

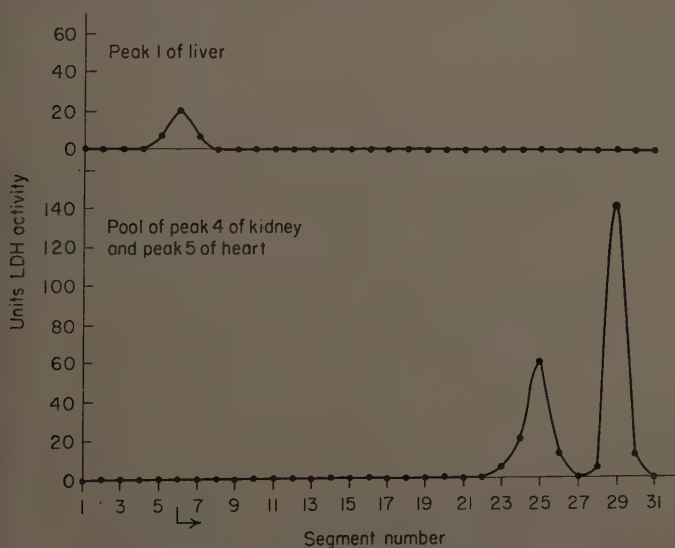


FIGURE 6. The patterns obtained when peaks isolated from a starch block were rerun on a second block. The upper curve represents peak 1 of liver homogenate, rerun on a second block. The lower curve represents a rerun of a pool of peak 4 from kidney homogenate, and of peak 5 from heart homogenate on a second block. Note that when these peaks are rerun they do not alter in mobility or give rise to peaks not present in the initial run. Reproduced by permission of *The Journal of Clinical Investigation*.

tient who had an acute attack of chronic relapsing pancreatitis associated with alcoholism there was an elevation of serum isozymes 1 and 2, which probably reflected liver damage. Electrophoretic separation of serum prior to LDH assay provides a greater specificity in the localization of pathology and appears to offer, therefore, an approach to the diagnosis of several diseases. Caution must

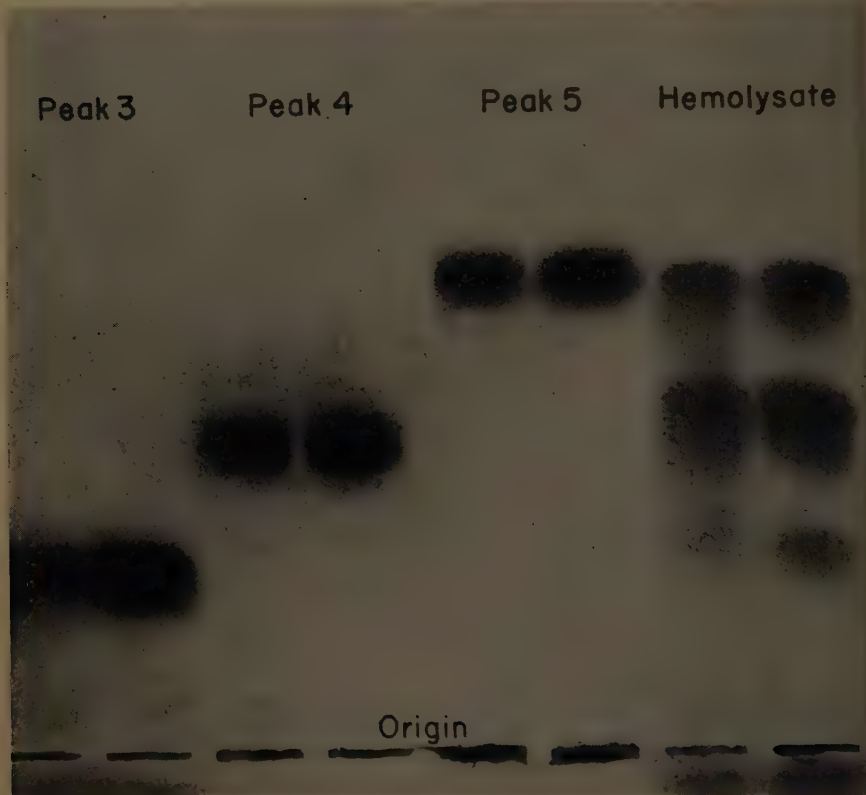


FIGURE 7. Distribution of lactic dehydrogenase activity in human hemolysate separated by starch-gel electrophoresis. Peaks 3, 4, and 5 isolated from human hemolysate separated on a starch block have been rerun on a Na_2HPO_4 -citric acid starch gel at pH 7.0, the isoelectric point of hemoglobin that remains at the origin. Note that under these conditions the peaks isolated from the block correspond in mobility to those obtained by separation of hemolysate on the gel; they do not give rise to additional peaks.

be exercised in the interpretation of the serum LDH isozyme pattern because the supervention of either shock or hemolysis in the patient may alter the pattern observed and therefore complicate the interpretation.

Recent comprehensive reports by Hess and Walter,¹² Hill,¹³ Weime,¹¹ and Wróblewski *et al.*¹⁰ are in general agreement with these results. However, Wróblewski *et al.*¹⁰ and Wieme,¹¹ utilizing starch- and agar-gel electrophoresis respectively, identified 5 LDH isozymes in normal serum, 1 more than had been observed employing starch-block and continuous-flow electrophoresis.¹⁻⁴

This additional isozyme was present in the γ globulin in trace amounts.¹¹ Either by concentrating the eluates from the starch block or by using less fluid to eluate the protein from the block we have confirmed the presence of this peak that normally accounts for approximately 2 per cent of the total serum LDH activity.

A wide variety of enzymes are known to exhibit different molecular forms in 1 organism and even in 1 tissue.²⁶⁻³⁵ Wieland and Pfeleiderer demonstrated several electrophoretically distinguishable isozymes of LDH in various organs of the rat⁵ and, in these pages, have offered evidence that these LDHs differed in their amino acid composition, the more rapidly migrating isozymes containing more acidic and less basic residues.³⁶ In human tissues the K_m and kinetic behavior with DPN analogs of isozymes 3, 4, and 5 were similar but varied from the K_m and DPN analog kinetics of isozymes 1 and 2, suggesting that the active centers of isozymes 3, 4, and 5 differed from the active centers of isozymes 1 and 2. Differences in electrophoretic mobility of isozymes 3, 4, and 5 may be explained by postulating structural differences in the enzyme other than at the active center. These differences could influence the intracellular location of an enzyme,³⁴ and could affect the binding of an enzyme to different cytoplasmic particles within a specialized cell. One gene might control the catalytic site, and other genes or the chemical environment of the cell might determine the attachment site of the enzyme.³⁷ This hypothesis may apply to the species differences in insulin, ACTH, and ribonuclease.³⁸ Recent studies of polymorphism in pseudocholinesterase reveal that the mutant enzyme differs from the normal type in pH optima and in affinity for different substrates.³⁹ In this case, the gene probably determines the polypeptide chain of the catalytic active center of the enzyme.

Summary

Five LDH isozymes were found in human tissues, and each of the homogenized human tissues examined electrophoretically exhibited heterogeneity of LDH activity. Each tissue had a constant number of activity peaks and a characteristic distribution of LDH activity in these peaks. The major portion of the LDH activity in heart homogenate was in peak 5, with a minor component in peak 4. Liver and skeletal muscle had greatest activity in peak 1; pancreas in peaks 3 and 5; lung in peaks 3 and 4, with some activity in peaks 1, 2, and 5; leukocytes and serum exhibited greatest activity in peak 4, whereas hemolysate and kidney had most activity in peaks 4 and 5.

Alterations of the serum LDH isozyme pattern in disease states correlated with the pattern of LDH isozymes in the damaged organ. In sera from cases of acute pancreatitis there was an elevation of peak 3, the peak containing most of the LDH activity in pancreas, whereas in hepatitis serum a large peak of LDH activity is described that is present only in trace amounts in normal serum and that had the same mobility as the peak in liver with greatest LDH activity. These observations suggest that assay of the LDH activity in the fractions of electrophoretically separated serum permits a greater specificity in localizing pathology than does assay of whole serum LDH activity. However, alterations in the serum LDH isozyme pattern can be produced by shock

or hemolysis, which may complicate the interpretation of the isozyme pattern observed in myocardial infarction, leukemia, hepatitis, and pancreatitis.

The use of DPN analogs and studies of Michaelis-Menten constants revealed that isozymes 1 and 2 exhibited different kinetic behavior from isozymes 3, 4, and 5, and suggested that the active centers of isozymes 1 and 2 differed from the active centers of isozymes 3, 4, and 5.

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References

1. VESELL, E. S. & A. G. BEARN. 1957. Localization of lactic acid dehydrogenase activity in serum fractions. *Proc. Soc. Exptl. Biol. Med.* **94**: 96.
2. VESELL, E. S. & A. G. BEARN. 1958. Observations on the heterogeneity of malic and lactic dehydrogenase in human serum and red blood cells. *J. Clin. Invest.* **37**: 672.
3. VESELL, E. S. & A. G. BEARN. 1961. Isozymes of lactic dehydrogenase in human tissues. *J. Clin. Invest.* **40**: 586.
4. SAYRE, F. W. & B. R. HILL. 1957. Fractionation of serum lactic dehydrogenase by salt concentration gradient elution and paper electrophoresis. *Proc. Soc. Exptl. Biol. Med.* **96**: 695.
5. WIELAND, T. & G. PFLEIDERER. 1957. Nachweis der Heterogenität von Milchsäuredehydrogenase verschiedenen Ursprungs durch Trägerelektrophorese. *Biochem. Z.* **329**: 112.
6. HESS, B. 1958. DPN-Dependent Enzymes in Serum. *Ann. N. Y. Acad. Sci.* **75**(1): 292.
7. PLAGEMANN, P. G. W., K. F. GREGORY & F. WRÓBLEWSKI. 1960. Electrophoretically distinct forms of mammalian lactic dehydrogenase. *J. Biol. Chem.* **235**: 2282.
8. MARKERT, C. L. & F. MØLLER. 1959. Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. *Proc. Natl. Acad. Sci. U. S. A.* **45**: 753.
9. KAPLAN, N. O., M. M. CIOTTI, M. HAMOLSKY & R. E. BIEBER. 1960. Molecular heterogeneity and evolution of enzymes. *Science*. **131**: 392.
10. WRÓBLEWSKI, F., C. ROSS & K. F. GREGORY. 1960. Isoenzymes and myocardial infarction. *New Engl. J. Med.* **263**: 531.
11. WIEME, R. J. 1959. Studies on Agar Gel Electrophoresis. Brussels, Arscia Uitgeverij N.V.
12. HESS, B. & S. I. WALTER. 1961. Chromatographic differentiation of lactate dehydrogenase of human tissue and serum. *Ann. N. Y. Acad. Sci.* **94**(3): 890.
13. HILL, B. R. 1961. Electrophoretic fractionation of serum lactic dehydrogenase. *Cancer Research*. **21**: 271.
14. DRABKIN, D. L. 1946. Spectrophotometric studies. XIV. The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species. *J. Biol. Chem.* **164**: 703.
15. HIRSCH, J. G. & A. B. CHURCH. 1960. Studies of phagocytosis of group A streptococci by polymorphonuclear leukocytes *in vitro*. *J. Exptl. Med.* **111**: 309.
16. FINE, J., H. A. FRANK, F. B. SCHWEINBURG, S. JACOB & T. GORDON. 1952. The bacterial factor in traumatic shock. *Ann. N. Y. Acad. Sci.* **55**(3): 429.
17. VESELL, E. S., M. P. FELDMAN & E. D. FRANK. 1959. Plasma lactic dehydrogenase activity in experimental hemorrhagic shock. *Proc. Soc. Exptl. Biol. Med.* **101**: 644.
18. KUNKEL, H. G. 1954. Zone Electrophoresis in *Methods of Biochemical Analysis*. **1**: 141. D. Glick, Ed. Interscience. New York, N.Y.
19. SMITHIES, O. 1959. Zone electrophoresis in starch gels and its application to studies of serum proteins. *Advances in Protein Chem.* **XIV**: 65.
20. POULIK, M. D. 1957. Starch-gel electrophoresis in a discontinuous system of buffers. *Nature*. **180**: 1477.
21. KUBOWITZ, F. & P. OTT. 1943. Isolierung und Kristallisation eines Garungsfermentes aus Tumoren. *Biochem. Z.* **314**: 94.
22. DEWEY, M. M. & J. L. CONKLIN. 1960. Starch gel electrophoresis of lactic dehydrogenase from rat kidney. *Proc. Soc. Exptl. Biol. Med.* **105**: 492.
23. WACKER, W. E. C., D. D. ULMER & B. L. VALLEE. 1956. Metalloenzymes and myocardial infarction. II. Malic and lactic dehydrogenase activities and zinc concentrations in serum. *New Engl. J. Med.* **255**: 449.

24. LINEWEAVER, H. & D. BURK. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**: 658.
25. NISSELBAUM, J. S. & O. BODANSKY. 1961. Reactions of human tissue lactic dehydrogenase with anti-sera to human heart and liver lactic dehydrogenases. *J. Biol. Chem.* **236**: 401.
26. KOWLESSAR, O. D., L. J. HAEFFNER & M. H. SLEISENGER. 1960. Localization of leucine aminopeptidase in serum and body fluids by starch gel electrophoresis. *J. Clin. Invest.* **39**: 671.
27. DESREUX, V. & R. M. HERRIOTT. 1939. Existence of several active components in crude pepsin preparations. *Nature*. **144**: 287.
28. KUNITZ, M. 1938. Formation of new crystalline enzymes from chymotrypsin. Isolation of beta and gamma chymotrypsin. *J. Gen. Physiol.* **22**: 207.
29. PALEUS, S. & J. B. NEILANDS. 1950. Preparation of cytochrome c with the aid of ion exchange resin. *Acta chem. Scand.* **4**: 1024.
30. HIRS, C. H. W., W. H. STEIN & S. MOORE. 1951. Chromatography of proteins. Ribonuclease. *J. Am. Chem. Soc.* **73**: 1893.
31. TALLAN, H. H. & W. H. STEIN. 1951. Studies on lysozyme. *J. Am. Chem. Soc.* **73**: 2976.
32. MALMSTRÖM, B. G. 1957. The purification of yeast enolase by zone electrophoresis and ion-exchange chromatography, and the existence of several active forms of the enzyme. *Arch. Biochem.* **70**: 58.
33. KOWLESSAR, O. D., J. H. PERT, L. J. HAEFFNER & M. H. SLEISENGER. 1959. Localization of 5-nucleotidase and non-specific alkaline phosphatase by starch gel electrophoresis. *Proc. Soc. Exptl. Biol.* **100**: 191.
34. BROMAN, L. 1959. Separation and characterization of two coeruloplasmins from human serum. *Nature*. **182**: 1655.
35. WHITTAKER, V. P. 1951. Specificity, mode of action and distribution of cholinesterases. *Physiol. Revs.* **31**: 312.
36. WIELAND, T. & G. PFLEIDERER. 1961. Chemical differences between multiple forms of lactic acid dehydrogenases. *Ann. N. Y. Acad. Sci.* **94**(3): 691.
37. LEVINTHAL, C. 1960. *Genetics*: 190. H. E. Sutton, Ed. Josiah Macy, Jr. Foundation. New York, N.Y.
38. ANFINSEN, C. B. 1959. *The Molecular Basis of Evolution*: 143. Wiley. New York, N.Y.
39. KALOW, W. & D. R. GUNN. 1959. Some statistical data on atypical cholinesterase of human serum. *Ann. Human Genetics.* **23**: 239.

CHROMATOGRAPHIC DIFFERENTIATION OF LACTATE DEHYDROGENASE OF HUMAN TISSUE AND SERUM*

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In 1958 it was reported by Hill,¹ Vesell and Bearn,² and one of the authors that LDH as well as other dehydrogenases can be obtained with specific heterogenous, enzymic, and physicochemical properties in serum of normal and pathological conditions. This was also observed by Wieme.⁴ Since it was obvious that the heterogeneity of serum LDH is a result of heterogenous tissue LDH, described in 1957 by Wieland and Pfeleiderer⁵ in rat tissues, it was suggested³ that the heterogeneity of serum dehydrogenases should generally be used for identification and differentiation of serum enzymes with respect to their cellular origin and should serve as a diagnostic indicator with high organ-specific properties, an approach originally also applied in case of the various alkaline phosphatases by Bodanski⁶ and Roche.⁷

In the meantime various authors added a great deal of information with respect to basic problems of heterogenous enzymes and also to pathological serum conditions, as we learn from the literature and from this monograph. In further continuation of our work and for a detailed study of human LDH we applied a chromatographic method using a DEAE column for isolation and differentiation of tissue and serum LDH.⁸⁻¹¹ Furthermore, a more practicable chromatographic batch technique was developed for quick differentiation in clinical routine procedure.¹² We here report the results of our study.

Methods and Materials

Column chromatography. Suitable dialyzed samples from serum or tissue and cell extracts (5 to 10 ml.) were chromatographed on DEAE-cellulose columns as earlier described in detail.¹¹ Starting with phosphate buffer (0.008 *M* pH 7.0) the elution was continued by increasing the ionic strength from 0.03 to 2.0 μ , using phosphate buffer of pH 6.0 to which NaCl was added in the later stages. The flow rate was 8 ml./hour. The effluent was collected in portions of 2.5 to 3 ml. in a fraction collector at +4° C. and checked for LDH activity. Also, a parallel analysis of the chromatographically separated serum proteins was made by paper electrophoresis, immunoelectrophoresis, ultracentrifugation, absorption spectra, and chemical analysis.¹⁰ We found a good reproducibility and an LDH activity yield of 90 to 100 per cent.

Batch technique. Two ml. serum or tissue extract (dialyzed against 0.02 *M* phosphate of pH 6.0) of a known LDH activity was mixed with 2 ml. DEAE-cellulose suspension (10 per cent in 0.02 *M* phosphate pH 6.0). After 10 min. of gentle stirring the mixture was centrifuged, and the LDH-activity was measured in the supernatant. The differences found before and after treatment with the DEAE cellulose show the amount of LDH activity absorbed.

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(within ± 5 per cent of the error). For exact calculation the dilution factor is taken into account (Hess and Walter¹²).

Determination of LDH activity. By means of the optical method, tests were performed either directly in the fraction volume or in an aliquot added to a test sample of phosphate buffer (0.2 *M*, pH 7.0) with a final volume of 2 ml. In both cases DPNH (0.5 μ M) and pyruvate (1.0 μ M) were added. The activity was expressed in Bücher units per milliliter and per milligram protein respectively.¹³ Protein was determined by the Biuret method according to Weichselbaum.¹³ The substrate affinity of LDH for pyruvate was calculated according to Lineweaver and Burk.¹⁴

Preparation of tissue and cell extracts and serum. Extracts from human organs were prepared as described earlier.¹¹ No significant loss in LDH activity was found in tissues between 24 and 72 hours post-mortem. Hemolysates from erythrocytes and extracts from thrombocytes were obtained according to Löhr and Waller.¹⁵ Serum was separated from fresh blood immediately after clotting. Hemolysis was carefully avoided. For column chromatography it was necessary to dialyze serum and extracts 4 hours at $+4^{\circ}$ C. against 3 l. of phosphate buffer (0.008 *M*, pH 7.0). For the batch technique a dialysis against 1 l. of phosphate buffer (0.02 *M*, pH 6.0) for 2 hours at room temperature was sufficient. Dialysis showed no significant effect on the LDH activity. A slight turbidity was always observed in the sera after dialysis and spun down in the centrifuge. It turned out to be a small amount of β and γ globulin (about 1 to 3 per cent of the total protein), which never showed LDH activity.

Reagents. In these experiments only deionized water was used. DEAE cellulose,* Na₂HPO₄·12 H₂O (p.a.), NaH₂PO₄·1 H₂O (p.a.), NaCl (p.a.), digitonin,† sodium pyruvate,‡ and DPNH§ were used.

Results

The chromatographic technique can be applied as a tool for characterization as well as for isolation of heterogenous proteins. The use of DEAE cellulose for protein fractionation, developed by Sober and Peterson,¹⁶ was found to be especially convenient for treatment of LDH proteins. In agreement with results obtained by the electrophoretic technique, 5 to 6 different fractions of LDH can be obtained from various sources. The feature of the structural pattern of LDH is defined by the number and chromatographic distribution of the fraction bands as well as by the ratios of their activities. Ninety per cent of the total activity of an LDH pattern is distributed between 1, 2, or 3 chromatographic locations. The given location of a band is independent of aging or heating (up to 56° C.). A mixture of 2 fractions can be rechromatographed and recovered without loss of activity at the original location.

Normal human serum contains a small LDH band that is found at the site of the LDH bands of erythrocytes (No. II or I), of thrombocytes (No. II) and

* Obtained from Serva-Entwicklungslabor, Heidelberg, West Germany.

† Purchased from Merck AG, Darmstadt, West Germany.

‡ From Hoffman-La Roche GmbH, Grenzach, Baden, West Germany.

§ From C. F. Boehringer and Son, GmbH, Mannheim, West Germany.

of heart muscle (No. II). Thus we cannot easily distinguish which one of these cells is the source of normal human serum LDH. Whereas heart muscle seems to be excluded because of its slow cellular turnover both blood cell types can well serve as a LDH source. Furthermore, leukocytes and intestinal mucosa must be considered.³

In pathological sera a variety of LDH patterns can be found in accordance

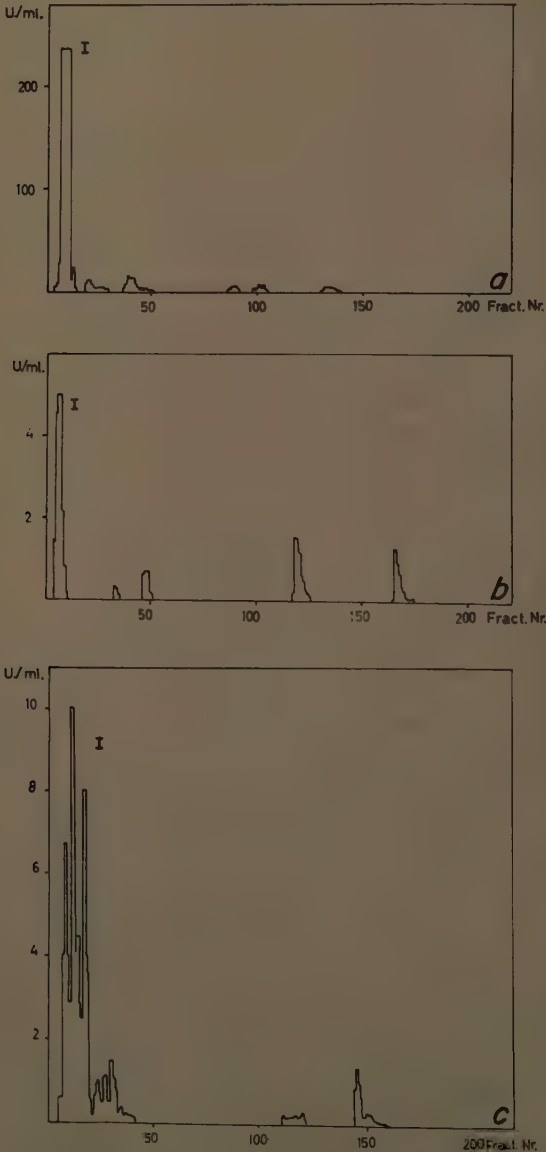


FIGURE 1. LDH pattern of human liver and sera of patients suffering from hepatitis (for details see text).

with data obtained by electrophoretic technique as demonstrated in greater detail elsewhere in these pages. FIGURE 1 summarizes the LDH pattern of normal human liver (*a*) and hepatitis sera (*b* and *c*). The major activity, it is found, is not absorbed by the DEAE cellulose and therefore collects in the first set of fraction tubes. In spite of the great lability of this LDH band a good recovery is obtained on isolation from liver extract. Also, the serum LDH band is found at the expected location in case of slight (*b*) or severe (*c*)

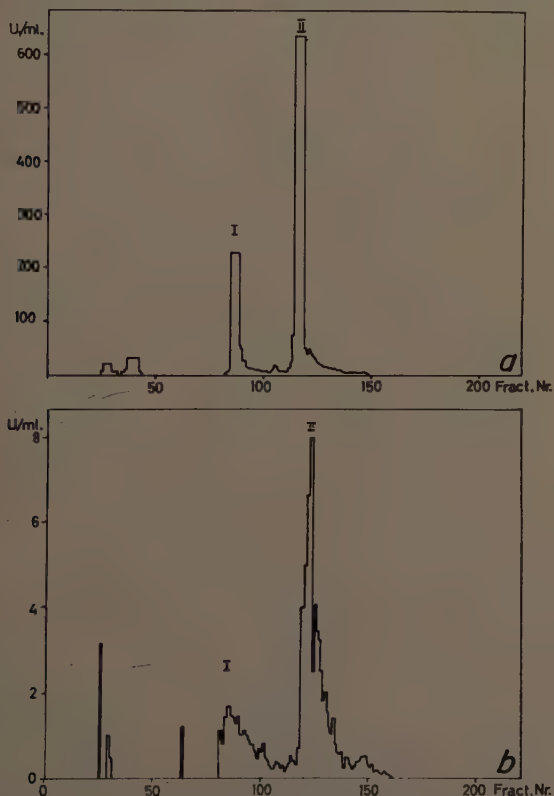


FIGURE 2. LDH pattern of human heart and serum of a patient with myocardial infarction (see text).

hepatitis. The additional LDH bands in *b* are identified as the blood cell type band and are probably due to hemolysis.

The LDH pattern of human heart and serum of a patient with myocardial infarction is shown in FIGURE 2. The LDH bands of human heart are strongly absorbed by DEAE cellulose, in contrast to those of liver LDH. The activity ratio of the two bands ($I < II$), as well as their location, is typical for this tissue. Thus the identification of the pattern does not raise problems of overlapping.

The LDH pattern of human blood cells is of special significance for identification of the pattern of normal serum. FIGURE 3 demonstrates a number of

typical LDH bands of human erythrocytes. Whereas a three band type (*a* and *b*) is found in a number of cases to correspond to the findings of Vese and Bearn² with the electrophoretic technique, a two-band LDH pattern of the same activity ratio ($I < II < III$, band I lacking) can also be found. A special difference in the three cases is not obvious. In case *a* erythrocytes were obtained from a patient with sprue. The samples were freshly taken and analyzed. In case *b* erythrocytes 10 days old from a blood bank sample

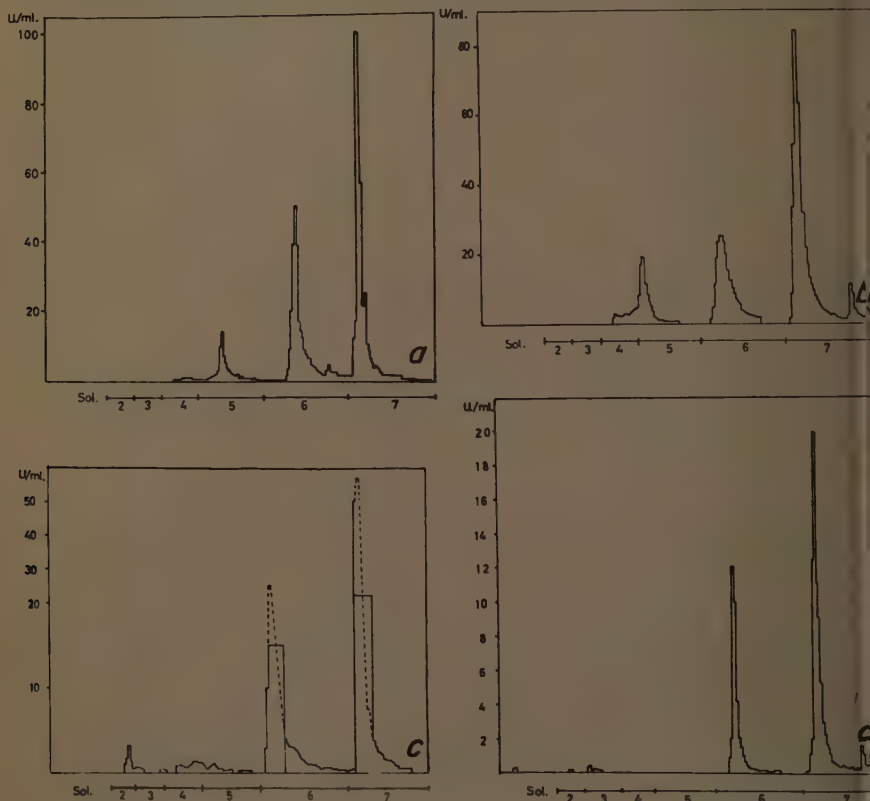


FIGURE 3. LDH pattern of human erythrocytes and serum of a patient with pernicious anemia (for explanation see text).

were analyzed. In case *c* a young normal person supplied the sample, which was also freshly analyzed. Finally, in case *d* a serum from a patient with pernicious anemia was analyzed, and a typical erythrocyte LDH pattern of the two-band type was found. As described earlier¹⁷ pernicious anemia is associated with a strong increase of LDH activity in serum. Summarizing erythrocytes may contain a two- or three-band pattern of LDH. A basis for an explanation of this LDH variety in a given cell type may be seen in genetic differences of the individuals, a hypothesis that needs further clarification.

A number of properties of LDH from human tissues and serum are gathered in TABLE 1, where the chromatographic location of LDH bands are related to

Origin	Chromatographic data						Corresponding location of serumprotein bands*
	No. of main peaks	Eluted from column by			Ratio of activities		
		pH	phosphate	NaCl		$\Gamma/2 = \mu$	
Liver	1: I.	7.0	0.008 M	—	0.03	—	γ Globulin
Serum of hepatitis patient	1: I.	7.0	0.008 M	—	0.03	—	
Myocard	2: I. II.	6.0 6.0	0.05 M 0.05 M	0.05 M 0.1 M	0.25 0.3	I < II	I. Albumin, α_1 , α_2 globulins
Serum of pat. with myocardial infarction	2: I. II.	6.0 6.0	0.05 M 0.05 M	0.05 M 0.1 M	0.25 0.3	I < II	II. Albumin, α_2 , β globulins
Erythrocytes type a	3: I. II. III.	6.0 6.0 6.0	0.05 M 0.05 M 0.1 M	0.05 M 0.1 M 0.1 M	0.25 0.3 0.5	I < II < III	I. Albumin, α_1 , α_2 globulins II. Albumin, α_2 , β globulins III. α_2 , β Lipoproteins β Macroglobulin
Erythrocytes type b	2: II. III.	6.0 6.0	0.05 M 0.1 M	0.1 M 0.1 M	0.3 0.5	II < III	
Serum of patient with perniciousa	2: II. III.	6.0 6.0	0.05 M 0.1 M	0.1 M 0.1 M	0.3 0.5	II < III	
Thrombocytes	3: I. II. III.	6.0 6.0 6.0	0.05 M 0.05 M 0.1 M	0.05 M 0.1 M 0.1 M	0.25 0.3 0.5	I > II > III	I. Albumin, α_1 , α_2 globulins II. Albumin, α_2 , β globulins III. α_2 , β Lipoproteins, β macroglob.
Kidney	1	6.0	0.05 M	0.1 M	0.3	—	II. Albumin, α_2 , β globulins
Skeletal muscle	2-3: I. II. III.	7.0 6.0 6.0	0.008 M 0.01 M 0.02 M	— — —	0.03 0.035 0.07	I \approx II	I. γ Globulin II. γ Globulin, β_1 globulin III. albumin, β_2 globulin

* According to data of paper electrophoresis, immunoelectrophoresis, and ultracentrifuge.

the corresponding serum proteins of the same fractions. The pattern of thrombocytes, kidney, and skeletal muscle are included. It is of interest that the chromatographic properties do not necessarily follow the rules observed with the electrophoretic technique. Thus the main fraction of albumin is eluted prior to the main band of heart LDH.

On the basis of the chromatographic properties a batch technique was developed. The aim of this method was not so much the pure isolation and complete characterization of several pure LDH than the "one-step" analysis and identification of the main LDH types from mixtures of these as it occurs

TABLE 2
CHROMATOGRAPHIC DIFFERENTIATION OF LDH BY BATCH TECHNIQUE

Exp. No.	Material	LDH activity in the sample						Error (% of total activity)	Remarks
		Total		Not adsorbed		Control-test			
		(I.U.)*	(%)	(I.U.)	(%)	Calculated I.U.	Found I.U.		
1	Serum, hemolytical anemia	0.99	100	0.11	11	0.88	0.90	+2	♀ 55 years old
2	Serum, hemolytical anemia	1.22	100	0.17	14	1.09	1.05	-3.3	♀ 75 years old
3	Serum, myocardial infarction	1.96	100	0.13	7	1.86	1.84	-1.2	♀ 60 years old
4	Serum, myocardial infarction	1.70	100	0.13	7	1.61	1.82	+12.4	♀ 26 years old reinfarction anterior-transmural
5	Serum, hepatitis epidemica	1.23	100	0.98	80	0.42	0.37	-4.1	♂ 57 years old acute
6	Serum, hepatitis epidemica	1.00	100	0.73	73	0.46	0.45	-1.0	♂ 61 years old acute
7	Serum, cirrhosis hepatitis decompensated	0.270	100	0.176	65	0.160	0.174	+5.2	♂ 60 years old
8	Erythrocyte hemolysate (human)	7.25	100	±0	±0	7.25	7.10	-2.1	Digitonin-hemolysate not diluted
9	Skeletal muscle extract (human)	5.20	100	4.20	80	2.28	2.40	+2.3	1:14 diluted

* I.U. = international units.

for example, in human blood, especially under pathological conditions. The importance of such findings for clinical problems increases with simplification of the method, which must, however, still include the possibility of a quantitative assay.

The results of this method are given in TABLE 2. Various percentages of nonadsorption are found in liver, heart, and erythrocytes LDH, and also in pathological sera containing heterogeneous LDH proteins. The simple test is useful for quick differentiation of LDH activity, for instance in jaundice due to a hemolytic syndrome or hepatitis.

A detailed discussion of the implications of the findings is given in Hess and Walter.¹¹

Summary

Chromatographic analysis of LDH protein in various human tissues and in sera of pathological conditions reveals a typical pattern of the LDH structure.

On the basis of typical chromatographic location, activity ratio, and number of protein bands, the origin of a given LDH activity can be identified.

For a quick identification of LDH proteins a chromatographic batch technique is described and applied to various samples. This method is also useful for identification of other proteins.

Acknowledgment

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References

1. HILL, B. R. 1958. Further studies of the fractionation of lactic dehydrogenase of blood. *Ann. N.Y. Acad. Sci.* **75**(1): 304.
2. VESELL, E. S. & A. G. BEARN. 1958. The heterogeneity of lactic and malic dehydrogenase. *Ann. N.Y. Acad. Sci.* **75**(1): 286.
3. HESS, B. 1958. DPN-dependent enzymes in serum. *Ann. N.Y. Acad. Sci.* **75**(1): 292.
4. WIEME, R. J. 1958. Studies on agar gel electrophoresis, techniques-applications, University of Ghent. Department of Internal Medicine. Ghent, Belgium.
5. WIELAND, T. & G. PFLEIDERER. 1957. Nachweis der Heterogenität von Milchsäuredehydrogenase verschiedenen Ursprungs durch Trägerelektrophorese. *Biochem. Z.* **329**: 112.
6. BODANSKI, O. 1937. Are the phosphatase of bone, kidney, intestine and serum identical? The use of bile acids in their differentiation. *J. Biol. Chem.* **118**: 341.
7. ROCHE, J. 1950. Phosphatases. *In* The Enzymes. I: Part 1, 473.
8. HESS, B. 1959. Serumfermente als Indikatoren zellulärer Funktionen in: Struktur und Stoffwechsel des Herzmuskels, von W. Hauss und H. Losse, Stuttgart, 1959 (nach dem I. Symposion an der Med. Univ. Klinik, Münster/Westf. 1958).
9. HESS, B. 1958. Untersuchungen über die Heterogenität der Serumfermente und ihre Verwendung als organspezifische Indikatoren zellulärer Funktionen. *Klin. Wochschr.* **36**: 985.
10. HESS, B. & S. I. WALTER. 1960. Chromatographische Serumweiß fraktionierung und ihre klinische Anwendung, *Verhandl. Deut. Ges. Inn. Med.* **66**: 639.
11. HESS, B. & S. I. WALTER. 1960. Über das Protein der Laktatdehydrogenase im menschlichen Serum und Geweben. *Klin. Wochschr.* **38**: 1080.
12. HESS, B. & S. I. WALTER. 1961. Adsorptionsmethode zur Differenzierung der Laktatdehydrogenase und anderer löslicher Fermente und Proteine im Serum und Geweben. *Klin. Wochschr.* **39**: 213.
13. BEISENHERZ, G., H. J. BOLTZE, T. BÜCHER, R. CZOK, E. GARBADE, E. MEYER-ARENDT & G. PFLEIDERER. 1953. Diphosphofructose aldolase, phosphoglyceraldehyde dehydrogenase, lactic acid dehydrogenase, glycerophosphate dehydrogenase, and pyruvate kinase from rabbit muscle in one process. *Z. Naturforsch.* **8b**: 555.
14. LINEWEAVER, H. & D. BURK. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**: 658.
15. LÖHR, G. W. & H. D. WALLER. 1959. Zellstoffwechsel und Zellalterung. *Klin. Wochschr.* **37**: 833.
16. SOBER, H. A. & E. PETERSON. 1954. Chromatography of proteins on cellulose ion-exchanger. *J. Am. Chem. Soc.* **76**: 1711.
17. HESS, B. & E. GEHM. 1955. Über die Milchsäuredehydrogenase im menschlichen Serum. *Klin. Wochschr.* **33**: 91.

THE FIFTH (ELECTROPHORETICALLY SLOWEST) SERUM LACTIC DEHYDROGENASE AS AN INDEX OF LIVER INJURY

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Introduction

Total lactic dehydrogenase activity (LDH) of normal human serum has been separated by different electrophoretic techniques into 5 fractions. In this paper we shall consider the significance of that LDH fraction that, at pH 8.4, migrates most slowly, and to this fraction we shall refer, according to conventional electrophoretic practice, as LDH5. Under *Enzymoelectrophoresis* below, we describe the technique of electrophoresis and of enzyme detection ("enzymoelectrophoresis") used in our studies. Under *Review of Data Concerning Human Serum LDH5* we review findings concerning LDH5 that already have been published, and, under *New Experimental Investigations* we report on new experiments done on rats and monkeys. It is concluded that direct experimental proof has been given of the relation between the serum level of LDH5 and the necrotic turnover occurring in liver cells.

Enzymoelectrophoresis

By this term we designate a special technique for the electrophoretic study of enzymes. To this end the mixture containing the enzymes is subjected to electrophoresis in an agar gel and, immediately afterward, enzyme activity is revealed by applying a spectrophotometric technique of enzyme assay over the whole length of the electrophoresis plate.¹ Enzyme activity can be followed by direct photometry, since an agar gel is transparent down into the ultraviolet of medium range.

In enzymoelectrophoresis we couple agar-gel electrophoresis to a Warburg type of enzyme assay. This is to be compared to immunoelectrophoretic techniques that combine agar-gel electrophoresis with an Ouchterlony type of immunoprecipitation.² It is because of this analogy that we coined the term enzymoelectrophoresis, using immunoelectrophoresis as model and excuse.^{3,4}

Enzymoelectrophoresis is not to be confused with histochemistry technique applied to an electrophoretic medium. A basic difference resides in the way substrates are introduced into that medium and, also, in the way enzyme kinetics are followed. In enzymoelectrophoresis, substrates are introduced by diffusion out of a second agar layer (of identical dimensions) laid on the electrophoresis plate after completion of the electrophoretic run (FIGURE 1). This has the advantage of minimizing broadening of the bands, since no elution occurs, as may be the case if the plate is simply bathed in a fluid solution containing the substrates. Furthermore, the twinned plate can easily be transferred into a spectrophotometer, and the kinetics of the enzyme reaction

plotted. Especially the conversion $\text{NAD} \rightleftharpoons \text{NADH}_2^*$ can easily be followed as an indicator of enzyme activity (spectral range 340 to 370 $\text{m}\mu$).

For electrophoresis proper, we use a technique of agar-gel electrophoresis previously described, which yields fractionations of outstanding quality.^{4,5} The sample to be analyzed is applied in a very narrow groove (length 4 mm., width one-fourth mm.) cut into the gel. Separation is achieved in about 25 min., since very high field strengths (30 v/cm.) are applied; under these conditions human albumin migrates over about 50 mm. Cooling and sealing of the plate against evaporation is obtained by immersing the gel in petroleum ether of low boiling point (25° to 50° C.). It is also very important to regularize the electroosmotic flow, which has a high intensity, by placing large agar blocks as bridges between the electrophoresis plate and the electrode cups. The whole experimental setup is kept simple by using microscope slides (0.9 × 25 × 76 mm.) covered with an agar layer of 1.5 mm. Thus the electrophoretic field is short and, with only 200 v between electrodes, a real high-voltage electrophoresis is realized. Since the electrophoretic field remains strictly homogeneous during the whole duration of the run, electrophoretic

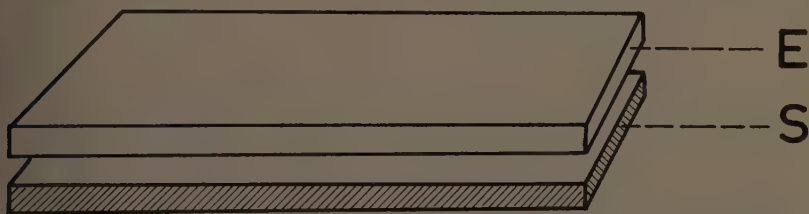


FIGURE 1. Basic features of enzymoelectrophoresis: two agar-gel plates of identical dimensions are brought into contact. Plate *E* is the electrophoresis plate (containing the enzymes separated by electrophoresis); *S* is the substrate plate.

mobilities can validly be measured. Generally we restrict ourselves to measurement of m_r (relative mobility) values, that is, *relative* electrophoretic mobilities: the ratio of the distance covered by the substance X to that of pure human albumin† under identical electrophoretic conditions. However, a recent modification in our technique⁶ assures rigid thermostatic control; hence measurements of *absolute* mobilities now can be made.

For the investigations we are dealing with, the electrophoretic separation was effected in an agar gel containing 1 per cent of Difco Special Agar-Noble (not especially purified; see further, however, under "*Practical Observations*," below) in a barbital buffer of pH 8.4 and ionicity 0.05. The size of the sample was 1.5 to 1.8 $\mu\text{l.}$, introduced with a graduated capillary into a small slit cut into the gel and carefully dried by sliding a piece of filter paper into the cut.

While the electrophoretic separation is progressing, the substrate plate is poured.^{4,7} For detection of LDH activity, it should contain pyruvate and NADH_2 . Good results are obtained with a plate prepared as follows. Into

* $\text{NAD} = \text{DPN}$ of the old nomenclature; $\text{NADH}_2 = \text{DPNH}$ of the old nomenclature. NAD is nicotinamide-adenine dinucleotide.

† Obtained from Behringwerke, Marburg, West Germany.

test tubes are pipetted 2 ml. of a 2 per cent solution of Difco Special Agar Noble in barbital buffer of pH 8.4 and ionicity 0.10. Those tubes constitute a stock for later determinations. If a substrate plate is to be poured, one of those tubes is taken, 0.5 ml. of bidistilled water is added, and the whole is heated in the water bath. When the gel has completely dissolved, 1.0 ml. of solution containing 2 mg./ml. of a commercial NADH_2^* is added; finally 0.1 ml. of a 0.1 *M* solution of sodium pyruvate is added.† The contents of the tube are then poured on a microscope slide that is carefully leveled and allowed to set for setting.

Upon completion of the electrophoretic run, the plate containing the sample is placed on top of the substrate plate in order to have a direct contact between both agar layers. One should be careful not to trap air bubbles between them. This twinned plate is immediately transferred to a sensitive spectrophotometer modified to provide scanning of the plate in steps of one-fourth mm. with an optical slit of one-fourth by three mm. Readings of optical density are taken at intervals of one-fourth or one-half mm., as rapidly as possible. A base line is obtained corresponding to the optical density as measured at each point of the twinned plate. This should have constant value over the whole length of the field. However within a few minutes the substrate plate diffuses into contact with the enzyme, and modifications in light absorption will become manifest at the zones presenting enzyme activity.

With LDH, the NADH_2 is oxidized into NAD and optical density (read at 340 or 366 $m\mu$) decreases; in the base line, negative peaks (dips) appear. By placing the light beam on one of these areas of changing optical density, kinetics of the reaction can be followed. More often we simply effect a second scanning after 30 or 35 min., the time that was found to be optimal. From the area enclosed between the dips, percentages between fractions can be calculated (calculation of *surface percentages* or S per cent; see Wieme⁴); alternatively a cut may be given in the gel at each minimum and, thus, the electrophoretic mobility of those fractions may be measured by comparing, after staining of the plate, the distances between the cuts to the distance covered by the reference albumin. After the final scanning, the plate is fixated in the usual way in an acid alcoholic solution, dried and stained for proteins. For more details the reader is referred to the original papers^{1,3,4,11,12} or to Wieme.⁸

Review of Data Concerning Human Serum LDH5

In 1957 Vesell and Bearn⁹ together with Sayre and Hill¹⁰ were the first to describe electrophoretic multiplicity of serum LDH in the human. The former used starch-block electrophoresis; the latter, paper electrophoresis for that fractionation. Neither mentioned the existence of the very slow LDH5 with which we are concerned.

At that time we were applying the newly described technique of enzyme electrophoresis¹ to analogous problems, and our observations were partially in variance with those results; they were communicated at the sixth Colloquium

* DPNH, Boehringer and Son, Mannheim, West Germany.

† Experiments have demonstrated that better results are obtained with a concentration of pyruvate that is inferior to the value recommended in previous publications; LDH5 especially is then better revealed.

on Protides of the Biological Fluids (Bruges, Belgium, May 1958).^{3,11} In normal human serum the dominant LDH activity is found at the level of the 3 fractions described by the above-mentioned authors; however in all normal human sera a fourth, slower LDH fraction is revealed by enzymoelectrophoresis and, in extracts of human tissues (among others, liver and cardiac muscle), 5 fractions are demonstrated. The pattern found in human-heart tissue, where the most rapid LDH dominates is quite different from that seen in human liver. It explains the typical shiftings described by Vesell and Bearn in myocardial infarction, in which the most rapid serum LDH becomes the dominant one.

At that time we had made some observations pointing to the presence in human serum of a fifth, very slow LDH whose mobility corresponds exactly to that of the LDH5 demonstrated in nearly all human tissues, especially in liver cells. By increasing the amounts of normal serum applied for analysis, this very low LDH could clearly be demonstrated and, at the Fourth International Congress on Biochemistry (Vienna, Austria, September, 1958),¹² we were able to communicate that in normal human serum a LDH5 is present; its concentration is, however, extremely low.

This low concentration may furnish the explanation for the fact that Englehardt-Gölkel *et al.*¹³ also failed to detect more than 3 LDH fractions on paper chromatograms of human serum.

In certain pathological conditions, different from those signaled by Vesell and Bearn, very clear-cut modifications of serum LDH5 occur, especially in acute hepatitis. Those modifications were studied in a paper dealing with the application of LDH enzymoelectrophoresis to the diagnosis of liver disease.⁷ In all 8 cases of acute hepatitis that were analyzed, profound modifications were seen in the level of LDH5, with percentages ranging from 35 to 52 per cent as compared to 1 to 8 per cent found in normal serum. In 1958 Vuylsteek and Wieme¹⁴ gave a typical illustration of the value of LDH enzymoelectrophoresis in medical diagnosis. In a patient in whom all symptoms (also total LDH assay in the serum and electrocardiographic evidence) suggested myocardial infarction, it was demonstrated by electrophoretic fractionation of serum LDH that this affection could not be involved.

A detailed study of the multiplicity of LDH as found in human serum and tissues was made in a study we devoted to the multiple aspects of agar-gel electrophoresis.⁴ The concentration of LDH5 is much higher in cord blood (after normal partus) than in blood of the adult (TABLE 1). No clear differences are manifest when plasma is used instead of serum. No strong modifications are seen when serum is stored for weeks at +4° C. In normal human urine, high percentages of LDH5 can be found in nearly one half of the individuals; in others that fraction is either absent or present in traces. In normal cerebrospinal fluid, only traces of LDH5 are found. LDH5 seems to be entirely absent from human red blood cells; here LDH1 and LDH2 strongly dominate; LDH4 makes up for a few percentages, while LDH3, being exactly congruent with the hemoglobin cannot be studied; these results are in agreement with observations published by Vesell and Bearn¹⁵ in a paper especially devoted to the multiplicity of malic dehydrogenase.

Different tissues were also studied, and our previous results confirmed (TABLE 1). Electrophoretic mobilities in serum and tissue value of corresponding

LDH fractions apparently are identical. The m_r was measured and found to be: 0.93 ($s = 0.014$), 0.69 ($s = 0.014$), 0.45 ($s = 0.010$), 0.22 ($s = 0.020$) and 0.02 ($s = 0.013$). Determinations were made on 11 serum samples.

Attention was drawn to the special position taken by the skin (cutis): in LDH activity is very high; no trace of LDH1 and LDH2 is found, while LDH3, LDH4, and LDH5 are present in nearly equal amounts. This distribution is similar to that found in skeletal muscle. The work cited⁴ ends with a chapter on the use of LDH enzymoelectrophoresis in clinical chemistry. Its value in the diagnosis of myocardial infarction and of hepatitis was confirmed and the simplicity of the analytical procedure stressed. We pointed to the fact that it becomes possible to demonstrate an eventual double origin for an increase of total LDH activity in serum. Indeed in patients presenting myocardial

TABLE 1
S PER CENT* AS OBSERVED ON DIFFERENT MATERIAL OF HUMAN ORIGIN†

	LDH1 (%)	LDH2 (%)	LDH3 (%)	LDH4 (%)	LDH5 (%)
Venous serum (adults) (11 samples)	23.2 $s = 5.3$	47.5 $s = 10.7$	18.5 $s = 3.4$	7.5 $s = 5.0$	3.2 $s = 3.1$
Cord blood (normal partus) (6 samples)	11.8	24.2	18.0	20.2	25.9
Cerebrospinal fluid (1 sample)	36	37	21	5	trace
Red blood cells (2 samples)	39	56	?	5	none
Heart muscle (5 samples)	35.0	25.6	11.8	16.4	10.8
Liver (6 samples)	2.0	4.3	11.0	27.0	55.7
Renal tissue (6 samples)	12.3	14.0	24.3	25.5	23.5
Placenta (1 sample)	12	18	15	30	25
Thymus (1 sample)	10	11	30	28	21
Skin (cutis) (2 samples)	none	none	31	31	38
Skeletal muscle‡ (2 samples)	4	7	21	27	41
Malign tumor tissue (plasmo-cytoma)	20	13	26	23	17

* Percentage between areas.

† Summarized from results published in Wieme.⁴

‡ This determination was effected in the course of the work described in this paper.

infarction of great extension we regularly found, in addition to the already classical increase of LDH1, a net increase of LDH5. This suggests that liver injury is concomitant to circulatory failure. FIGURE 2 shows a diagram obtained 48 hours after onset of myocardial infarction in a patient presenting systolic blood pressure inferior to 8 cm. mercury.

In another work¹⁶ it was stressed that the multiplicity of LDH could be seen even in a homogeneous population of cells. In this connection the multiplicity of LDH as found in red blood cells is of special interest. Strictly speaking cannot be stated that one LDH is specific for liver and one for heart muscle; what is typical is the ratio between the different LDHs.

Lowenthal *et al.*¹⁷ discussed at the eighth Colloquium on Protides of the Biological Fluids (Bruges, May, 1960) the results they obtained by LDH enzymoelectrophoresis on normal and pathological cerebrospinal fluid; the concentration of LDH5 is very low. Extracts of the central nervous system were also analyzed for their LDH composition. Electrophoretic mobilities were found

to be very similar to those we measured under identical electrophoretic conditions in other human tissues. The percentages of those LDHs are shown in TABLE 2.

In 1960 Wróblewski *et al.*,¹⁸ using starch gel electrophoresis, also demonstrated the presence of 5 LDH fractions* in normal human serum. Electrophoretic mobilities seem to correspond roughly with those observed in an agar gel, indicating that the molecular dimensions of those enzymes cannot be markedly different. Some discrepancies however are to be noted as to the percentage distributions found in normal human serum and in different human tissues: the

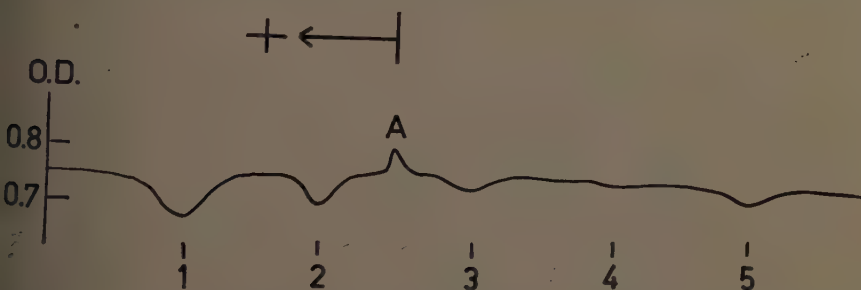


FIGURE 2. LDH enzyme electrophoresis of human serum (barbital buffer, pH 8.4, ionicity 0.05). In A, the line of sample application, LDH1 and LDH5 have increased. It was obtained in a patient presenting myocardial infarction with sustained low blood pressure. From the diagram the following S percentages were calculated: LDH1 45 per cent, LDH2 25 per cent, LDH3 10 per cent, LDH4 5 per cent, and LDH5 17 per cent.

TABLE 2
S PER CENT OBTAINED BY LDH ENZYMOELECTROPHORESIS*

	LDH1 (%)	LDH2 (%)	LDH3 (%)	LDH4 (%)	LDH5 (%)
Cerebrospinal fluid	38.5	30.0	24.3	7.1	5.1
Cerebral tissue:					
White matter	21.3	27.6	25.7	19.4	7.0
Grey matter	20.8	24.1	26.6	20.1	8.6

* Adapted from Lowenthal *et al.*¹⁷

weakest fraction in normal human serum should be LDH4; in human kidney, no LDH5 seems to have been demonstrated; LDH4 and 5 should only be present in traces in human-heart muscle. At any rate, the cited authors experience some difficulty in recovering LDH activity from the electrophoretic medium, since only 50 to 70 per cent of the total plasma LDH activity was found returned. Their paper further reports on the modifications seen in serum LDH patterns in patients presenting myocardial infarction.

The composite character of human LDH also became apparent when other than electrophoretic techniques were applied. In 1958 Hess¹⁹ demonstrated that human lactic dehydrogenase of different origin is different as to its pH

* For simplicity's sake we adapted their sequence in the numbering of LDH fractions to that used in this paper.

optimum and its substrate affinity. The application of these differences for the specific detection of cellular injury was suggested.

Hess and Walter²⁰ also demonstrated heterogeneity by column chromatography on DEAE cellulose. Elution with a phosphate buffer (pH 7 to 6), at increasing concentrations, first delivers an LDH as found predominantly in liver tissue; the main activity present in heart muscle is eluted only at high ionicities. In cases of hepatitis the main LDH activity of serum is eluted at low ionicities, as in the case of LDH of liver origin. This "liver LDH" seems to correspond within certain limits to our LDH5. However, results of chromatographic fractionation are not directly congruent to those obtained by electrophoresis.

Of special interest is the work of Rosalki and Wilkinson²¹ on differences between the serum LDH fractions as to their action on α -ketobutyrate: the slowest LDH fractions are much more active against pyruvate than against α -ketobutyrate, while the two most rapid ones are only slightly more active.

New Experimental Investigations

(1) *Choice of experimental procedure.* In the preceding paragraphs evidence was presented indicating a direct relationship between serum LDH5 and hepatic tissue. Direct experimental proof was still lacking. To this end carbon tetrachloride—a substance recognized to be hepatotoxic—was administered to rats and monkeys, and the shiftings occurring at the level of the serum LDHs were followed. Carbon tetrachloride was administered by intubation, a simple and safe route minimizing local necrotic action and affording precise knowledge of the actual dose.

Experiments were first performed on rats. Indeed we had made extensive investigations on the multiplicity of LDH in rats and mice^{12,22,4} (see also Wiland and Pfeleiderer;²³ and Pfeleiderer and Jeckel²⁴). In most tissues 5 LDH fractions were demonstrated by enzymoelectrophoresis; in liver tissue the slowest one strongly dominates, as in the human liver. In the serum of rats 5 LDHs may also be revealed; they have mobilities identical to those of the corresponding LDH fractions of the tissues, but they are quite different from the human LDHs ($m_r = 0.91, 0.80, 0.45, 0.28, \text{ and } 0.14$, determined in agar Difco Noble 1 per cent in barbital buffer, pH 8.4, and ionicity 0.05). Percentage distribution is even more different: in all normal rats the slowest LDH strongly dominates; in nearly all of them LDH4 is also easily demonstrated, while LDH1, LDH2, and LDH3 are revealed in a few sera only. Due to these rather large individual variations, we restricted ourselves to measuring the modifications that occur in the *area* enclosed by the peak corresponding to LDH5. In fact it is this peak that is suspected to reflect liver necrosis; furthermore, all our observations on rats to which carbon tetrachloride was given point toward modifications occurring mainly at that level. If absolute values are to be conferred to the measurement of that area, one should be careful to apply the samples in equal amounts and to scan the plates after identical time intervals; all other factors (especially temperature) should further be maintained as constant as possible.

For reasons discussed below, experiments on rats did not prove as satisfactory as could be expected. We therefore started experiments on *cinomolgus* mon-

keys (*Macaca irus*). These Primates proved to present a rather intriguing similarity to human beings in the multiplicity of their LDHs. In plasma, 5 fractions are easily demonstrated whose mobilities are identical, in so far as we can measure, to those of the corresponding fractions in human serum. This point needs to be stressed, since conventional serum proteins are clearly different from that point of view. Whether this similarity is a fortuitous fact or reveals some fundamental biological property related to the evolution of

TABLE 3

DISTRIBUTION OF LDH FRACTIONS IN PLASMA AND SOME TISSUES IN NORMAL MONKEYS (*CINOMOLGUS*)

	LDH1 (%)	LDH2 (%)	LDH3 (%)	LDH4 (%)	LDH5 (%)
Plasma:					
Individual 1	11.5	14.6	34.5	26.8	12.6
Individual 2	6.8	21.0	21.2	20.3	30.5
Individual 3	16.3	19.2	26.0	31.3	9.6
Individual 4	19.2	23.7	28.9	22.0	6.0
Individual 5	11.2	16.5	26.4	30.7	15.2
Individual 6	5.3	11.3	24.0	41.7	17.3
Mean	11.7	16.7	26.8	28.8	15.2
Tissues:					
Red blood cell					
Individual 1	24.6	50.8	17.3	7.2	0
Individual 2	33.2	46.6	12.4	7.6	0
Mean	28.9	48.7	14.8	7.4	0
Heart muscle					
Individual 1	35.1	16.3	14.4	16.1	18.2
Individual 2	35.5	23.8	12.4	13.7	14.6
Mean	35.3	20.0	13.3	14.9	16.4
Liver tissue					
Individual 1	39.2	9.7	9.8	28.0	43.3
Individual 2	5.6	6.8	16.1	28.4	43.0
Mean	7.4	8.2	12.9	28.2	43.1
Lung tissue	18.1	23.6	18.1	17.4	22.7
Spleen	12.5	15.2	14.9	22.9	34.2

species²⁵ remains an obscure but challenging question. As to the percentage distribution of these LDHs, here also some similarity is found, but global activity is higher and individual variations are more pronounced (see TABLE 3). For results obtained on different tissues, compare TABLE 3 with TABLE 1. Here too LDH5 is strongly predominant in liver but absent from red blood cells.

(2) *Carbon tetrachloride administration to rats.* Three groups, each of 8 individuals (male, adult, albino rats, inbred strain, kept on a standard diet), were studied. To the members of the first group a total amount of 0.6 ml. of

carbon tetrachloride was intubated in 3 doses of 0.2 ml. at intervals of 48 hours; the second group received a total amount of 1.2 ml. in 6 doses of 0.2 ml. intubated at the same intervals. The third group constituted controls that were not intubated.

The rats were beheaded 48 hours after the last dose, their blood collected in heparin, and the plasma separated by centrifugation. A check was made for the absence of discernible traces of hemolysis. The samples were then subjected to an assay for their oxaloacetic (SGOT) and pyruvic (SGPT) transaminase activity according to a conventional colorimetric procedure.* For enzyme electrophoresis, samples of 1.6 μ l. ($\pm 0.1 \mu$ l.) were applied to the electrophoresis plate (2 for each plate) and readings taken exactly 30 and 35 min. after pairing of the electrophoresis and substrate plate. All determinations were made at room temperature, 18 to 20° C. The area enclosed by the LDH5 peak was measured by transferring the curve on Whatman No. 1 paper, cutting it out and weighing (to 0.01 mg.). Since for all samples the determinations were made in duplicate, it was possible to obtain for each of them a scanning after 30 and 35 min.; the values tabulated correspond to the mean of both measurements.

The results are shown in TABLE 4. The area of the LDH5 peak significantly increases after the administration of carbon tetrachloride. Such increase amounts to 3.9 times the standard error of the difference after 0.6 ml., and 2.6 times this value after 1.2 ml.

The fact that hepatic necrosis was actually produced was proved by microscopic examination of the liver tissue that was selected from most animals. In all samples, extensive necrotic lesions were observed. Hepatic necrosis was also revealed by the net increase of SGOT and, especially, SGPT levels. It is to be noted that the largest dose did not provoke the highest levels in SGOT, SGPT, nor in LDH5. We deal with that point under *General Comments*.

If the increase of LDH5 after administration of carbon tetrachloride is statistically significant, it cannot be called dramatic. Enzyme electrophoresis suffers here from an apparent lack of sensitivity that can be explained on the following basis. In enzyme electrophoresis, kinetics are largely determined by the diffusion of the substrates. We found⁴ that the area enclosed between the peaks is a logarithmic function of enzyme concentration; as enzyme concentration goes up, the surface of the area as measured by enzyme electrophoresis increases less and less rapidly. Thus when studying LDH5 in rat plasma one is dealing with a fraction already present in high concentration; an increase of that concentration will induce only a small change in the corresponding area.

(3) *Carbon tetrachloride administration to monkeys.* In *cinomolgus* monkeys conditions are quite different. Here LDH5 is the weakest fraction in the normal subject. Also those animals proved extremely sensitive to the administration of carbon tetrachloride. Furthermore, experiments were made easier by the fact that the blood can repeatedly be sampled on the same animal by puncture of an artery or vein in the inguinal region.

Experimental procedures were identical to those described for the rats. The main experiments were performed on 2 male individuals of 1.1 kg. and 1.2 kg.

* We followed the procedure of the Sigma Chemical Co., St. Louis, Mo., Technical Bulletin No. 505.

weight. The effects of a single or of 2 successive doses (interval of 24 hours) of 0.5 ml. carbon tetrachloride given by intubation were studied.

The results are shown in TABLE 5. Twenty-four hours after a single small dose of 0.5 ml. carbon tetrachloride, LDH5, initially the weakest fraction,

TABLE 4
RESULTS OF SOME ENZYME ASSAYS IN RATS*

Animal	Area of LDH5 peak (arbitrary units)	SGOT (U./ml. plasma)	SGPT (U./ml. plasma)
Series: 0.6 ml. CCl ₄			
CC 15	2517	990	300
CC 16	2633	2000	1300
CC 17	2620	1070	360
CC 18	2986	1600	760
CC 19	3585	450	600
CC 20	2692	1500	870
CC 21	2762	730	300
CC 22	3402	1600	870
Arithmetic mean	2900	1240	670
	s = 360		
Series: 1.2 ml. CCl ₄			
CC 30	1825	270	72
CC 31	2800	920	335
CC 32	2535	225	81
CC 33	3025	470	74
CC 34	3115	1020	760
CC 35	2965	570	59
CC 36	2375	400	900
CC 37	2350	400	900
Arithmetic mean	2624	534	397
	s = 400		
Control series			
CO 3	2390	370	41
CO 4	1394	580	33
CO 7	1702	345	25
CO 8	2115	470	36
CO 13	1430	270	33
CO 14	1470	320	48
CO 23	2674	490	33
CO 24	2895	370	30
Arithmetic mean	2008	402	34.9
	s = 590		

* Forty-eight hours after carbon tetrachloride (total dose of 0.6 ml. or 1.2 ml.); a control series is also included.

steeply rises and accounts for more than 50 per cent of the total LDH activity (FIGURE 3). These results strongly recall what is seen in patients with hepatitis. However, if the dose is repeated the next day, this is not followed by a new increase; on the contrary, the level is inferior to that of the preceding determination. This evokes what was seen in rats, and will be considered below under *General Comments*. A few days after the last dose, the figures are normalized again.

The animals did not seem affected in any way by the small amounts that were given, but bioptic studies were not made of the status of the liver parenchyma.

Practical Observations

(1) Serum LDH5 seems more labile than the rapid ones. Indeed in some of our experiments we had difficulty in detecting that fraction although, in a

TABLE 5
EVOLUTION OF SERUM LDHS IN TWO MONKEYS (*CINOMOLGUS*) RECEIVING
SMALL DOSES OF CARBON TETRACHLORIDE

Time after first intubation of CCl ₄	LDH1 (%)	LDH2 (%)	LDH3 (%)	LDH4 (%)	LDH5 (%)
Animal K (24 hours before first experiment)	16.0	30.7	26.6	19.0	7.7
0 hour: 0.5 ml. CCl ₄					
24 hours	6.7	10.4	13.0	11.3	58.3
24.15 hours: 0.5 ml. CCl ₄					
48 hours	20.9	29.9	22.0	12.4	14.7
3 days	14.1	17.2	31.5	32.2	6.0
5 days	21.0	30.0	23.1	15.7	9.9
9 days: 0.5 ml. CCl ₄					
10 days	7.3	5.5	12.1	28.1	46.8
21 days	15.1	29.1	23.4	21.9	10.4
Animal N (24 hours before first experiment)	13.4	20.7	23.4	24.1	18.4
0 hour: 0.5 ml. CCl ₄					
24 hours	7.0	15.6	9.4	23.3	44.5
24.15 hours: 0.5 ml. CCl ₄					
48 hours	15.3	24.9	24.3	17.3	18.1
3 days	26.3	29.3	16.2	10.2	18.0
5 days	11.3	17.1	28.9	20.5	22.2
9 days: 0.5 ml. CCl ₄					
10 days	2.2	3.4	7.0	21.4	65.9
21 days	10.1	18.8	22.7	26.7	21.7

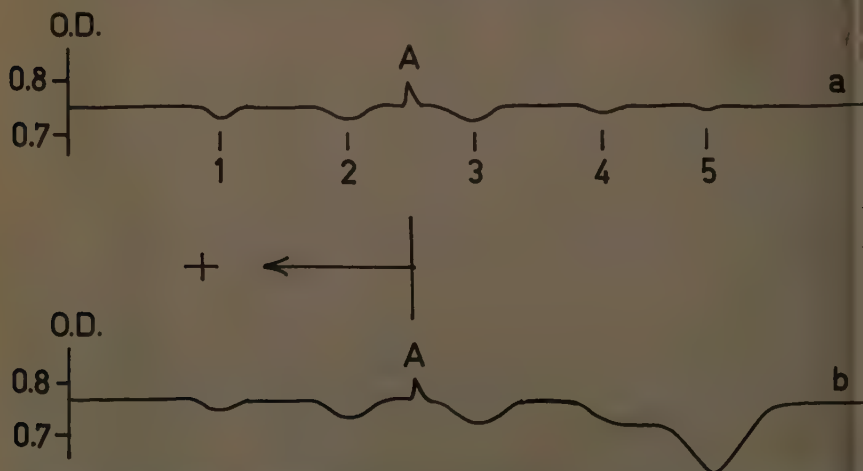


FIGURE 3. LDH enzyme electrophoresis of monkey (*cinomolgus*) plasma (barbital buffer pH 8.4, ionicity 0.05). Key: A, line of sample application; a, 24 hours before; and b, 24 hours after, intubation of 0.5 ml. CCl₄. Note steep increase of LDH5.

other runs, it was easily revealed. Finally it was demonstrated that the brand of agar used for the enzyme electrophoresis had some effect. With a certain batch of Difco Special Agar-Noble (that afterward proved quite suitable for simple electrophoretic experiments) some interaction occurred with LDH5, so that no clear-cut fraction was found at the usual place. All troubles vanished by simply switching to another batch of the same agar brand. We tried to purify the "bad" agar by repeated washing in distilled water, without noteworthy improvement.

(2) Since very small volumes of serum are used for enzyme electrophoresis, one could be tempted to select the sample by finger puncture. However, in this way a factor is introduced that can scarcely be controlled: admixture of LDH from the skin. Too high a figure will be found for LDH5.

(3) Hemolysis may be very disturbing, especially if one is studying LDH in cases of neoplastic disease or of myocardial infarction. However if one is interested in LDH5 in itself, hemolysis will do no harm since red blood cells do not possess that fraction. However, hemolytic samples should be rejected if percentages are to be calculated.

General Comments

Our studies do not preclude the possibility that necrosis occurring in *other tissue types* will not influence the level of LDH5. Especially necrosis of the skeletal muscle and of the skin could preferentially increase LDH5 since, in these tissues, that fraction is also predominating. However, it is probable that richness of the tissue in LDH and the pattern of its multiplicity do not constitute the sole factors determining the impact of necrosis on the serum LDH pattern: the nature of the barrier interposed between the tissue proper and the blood stream may also intervene.

Attention is drawn to the great difference observed as to the level of LDH5 in initial and later stages of liver necrosis: even if the necrogenic substance is continued in a dosage unchanged, the response at the level of LDH5 tends to decrease, in rats as well as in monkeys. The same tendency is found in the levels of SGOT and SGPT. This may be explained in the following way. In a first step, the LDH contained in the liver cells is liberated into the blood stream. Afterward the blood level falls, since the rate of synthesis of these proteins becomes the limiting factor.

Conclusions

From clinical evidence, from study of the LDH multiplicity in different tissues and, finally, from experiments on rats and especially on monkeys, it is concluded that necrosis occurring in the liver induces, at least in the first days, an increase of serum LDH5.

In these experiments, a falling level of LDH5 is found, even if the hepatotoxic substance continues to be given. This might be explained by exhaustion of the LDH reserves in the cells, followed by a second stage where the rate of LDH synthesis becomes the limiting factor determining the blood level of that protein.

Summary

The electrophoretic multiplicity of lactic dehydrogenase can easily be studied by agar-gel electrophoresis. To this end we applied a technique we called

enzymoelectrophoresis, described above. Of relative simplicity, this procedure yields percentages between peaks obtained on the diagrams and, also, affords calculation of electrophoretic mobilities.

In normal human serum we demonstrated 5 electrophoretic LDH fractions against the 3 described in 1957 by other authors. The slowest of them (we refer to it as LDH5), first described in 1958, is of special clinical interest since some relationship seems to exist with the status of the liver parenchyma: in acute hepatitis a very steep increase of serum LDH5 is seen.

To gain direct experimental evidence concerning this relationship, carbon tetrachloride was intubated to rats and cynomolgus monkeys. It was observed that the level of plasma LDH5 increases after the first doses. However, this level tends to decrease if the same hepatotoxic substance is continued to be given. An explanation is proffered.

In these experiments, cynomolgus monkeys appeared to constitute very suitable subjects, as they present an intriguing similarity to human beings in regard to the multiplicity of their LDH.

It is concluded that the level of serum LDH5 can be used as an index for liver necrosis, especially in the first days following the action of some hepatotoxic factor.

Acknowledgment

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References

1. WIEME, R. J. 1958. Über einige Aspekte der Elektrophorese im Agar-Gel (Vortrag an Behringwerken 13/3/57). Behringwerk-Mitteilungen. **34**: 233.
2. GRABER, P. & C. A. WILLIAMS. 1955. Méthode immuno-électrophorétique d'analyse de mélanges de substances antigéniques. Biochim. et Biophys. Acta. **17**: 67.
3. WIEME, R. J. 1959. Applications diagnostiques de l'enzymo-électrophorèse des déshydrogénases de l'acide lactique. Protides biol. Fluids (Proc. 6th Coll., Bruges, May 1958). : 236. Elsevier. Amsterdam, The Netherlands.
4. WIEME, R. J. 1959. Agar Gel Electrophoresis; Techniques, Applications. Arscid Brussels, Belgium.
5. WIEME, R. J. 1959. An improved technique of agar-gel electrophoresis on microscopical slides. Clin. Chim. Acta. **4**: 317.
6. WIEME, R. J. 1961. Assurance of thermostatic control during electrophoretic experiments. Nature. **190**: 806.
7. WIEME, R. J. & L. DEMEULENAERE. 1959. Enzymo-électrophorèse de la déshydrogénase de l'acide lactique, test sélectif d'intégrité parenchymateuse. Acta gastroenterolog. belg. **22**: 69.
8. WIEME, R. J. 1961. Agar Gel Electrophoresis. Elsevier. Amsterdam, The Netherlands.
9. VESELL, E. S. & A. G. BEARN. 1957. Localization of lactic acid dehydrogenase activity in serum fractions. Proc. Soc. Exptl. Biol. **94**: 96.
10. SAYRE, F. W. & B. R. HILL. 1957. Fractionation of serum lactic dehydrogenase by sucrose concentration gradient elution and paper electrophoresis. Proc. Soc. Exptl. Biol. **96**: 695.
11. WIEME, R. J. 1959. Applications diagnostiques de l'enzymo-électrophorèse des déshydrogénases de l'acide lactique. Clin. Chim. Acta. **4**: 46.
12. WIEME, R. J. 1958. IV Intern. Congress of Biochemistry (section 5, comm. 8) (Wien, September 1958). Pergamon Press. London, England.
13. ENGLHARDT-GÖLKEL, A., R. LÖBEL, W. SEITZ & I. WOLLER. 1958. Über das Verhalten und die Herkunft glykolytischer Serumenzyme beim Menschen und ihre diagnostische Bedeutung. Klin. Wochschr. **36**: 462.

4. VUYLSTEEK, K. & R. J. WIEME. 1958. Een geval van benigne pericarditis. Belg. Tijdschr. Geneesk. **14**: 750.
5. VESELL, E. S. & A. G. BEARN. 1958. Observations on the heterogeneity of malic and lactic dehydrogenase in human serum and red blood cells. J. Clin. Invest. **37**: 672.
6. WIEME, R. J. 1960. Sur la multiplicité de la lacticodéhydrogénase sérique chez l'homme. Proceedings of the I Colloquium on Enzymes in Clinical Chemistry, Ghent, April 1960. : 99. Arscia. Brussels, Belgium.
7. LOWENTHAL, A., D. KARCHER & M. VAN SANDE. 1961. Protides biol. fluids (Proc. 8th Coll., Bruges, May 1960). : 108. To be published. Elsevier. Amsterdam, The Netherlands.
8. WRÓBLEWSKI, F., C. ROSS & K. GREGORY. 1960. Isozymes and myocardial infarction. New Engl. J. Med. **263**: 531.
9. HESS, B. 1958. Untersuchungen über die Heterogenität der Serumfermente und ihre Verwendung als organspezifische Indicatoren cellularer Funktionen. Klin. Wochschr. **36**: 985.
10. HESS, B. & S. I. WALTER. 1960. Über das Protein der Laktatdehydrogenase im menschlichen Serum und Geweben. Klin. Wochschr. **38**: 1080.
11. ROSALKI, S. B. & J. H. WILKINSON. 1960. Reduction of α -ketobutyrate by human serum. Nature. **188**: 1110.
12. WIEME, R. J. 1959. Etude par enzymo-électrophorèse des déshydrogénases de l'acide lactique et de la déshydrogénase du sorbitol dans divers tissus chez le rat et la souris blanche. Bull. Soc. Chim. Biol. **41**: 235.
13. WIELAND, TH. & G. PFLEIDERER. 1957. Nachweis der Heterogenität von Milchsäuredehydrogenasen verschiedenen Ursprungs durch Trägerelektrophorese. Biochem. Z. **329**: 112.
14. PFLEIDERER, G. & D. JECKEL. 1957. Individuelle Milchsäuredehydrogenasen bei verschiedenen Säugetiere. Biochem. Z. **329**: 370.
15. KAPLAN, N. O., M. M. CIOTTI, M. HAMOLSKY & R. E. BIEBER. 1960. Molecular heterogeneity and evolution of enzymes. Science. **131**: 392.

LACTIC DEHYDROGENASE ISOZYMES AND THEIR DISTRIBUTION IN NORMAL TISSUES AND PLASMA AND IN DISEASE STATES*

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The lactic dehydrogenases from each of a number of mammalian species have been resolved into several electrophoretically and/or chromatographically distinct, enzymatically active forms of isozymes.¹⁻⁸ Human and rabbit tissues were each found to contain five of these isozymes.^{4,6,7,8} Some tissues yielded only one isozyme while others contained two, three, four, or all five forms in characteristic proportions. No two tissues studied were found to have identical lactic dehydrogenase (LDH) isozyme patterns. These isozymes were found to differ from each other in several properties in a regular manner that could be correlated with their electrophoretic mobilities in starch gel.^{9,10} Normal human plasma was found⁸ to have a characteristic distribution of LDH isozyme activities in which $LDH_2 < LDH_1 < LDH_3 < LDH_4 < LDH_5$ where the subscripts 1 to 5 refer to increasing electrophoretic mobilities. Myocardial infarction resulted in a marked increase in the proportion of LDH_5 , the isozyme present in heart muscle in highest concentration. The plasma isozyme pattern was found to be a more sensitive, specific, and lasting parameter of myocardial necrosis than the total serum or plasma enzyme activity.

In this paper previously published data on the regular differences between rabbit LDH isozymes are reevaluated, and further evidence of uniform differences in their properties is presented. Experiments on the LDH isozyme distribution in rabbit plasma are described. Studies on the plasma LDH isozyme patterns of 300 normal persons and 500 hospital patients with a wide variety of diseases are also reported.

Properties of Rabbit LDH Isozymes

Pyruvate concentration optima. All of the rabbit LDH isozymes, when catalyzing the reduction of pyruvate to lactate, are inhibited by increasing pyruvate concentrations that are still well below those theoretically required to allow the reaction to proceed near its maximum velocity as calculated from Lineweaver-Burk double reciprocal plot.⁹ Owing to this inhibition by excess pyruvate each isozyme has a pyruvate concentration optimum. This optimum was found to be strongly pH and temperature-dependent, decreasing with decreasing pH⁹ and decreasing temperature.¹⁰ At any fixed pH and temperature, the greater the electrophoretic mobility of an LDH isozyme the lower was the apparent Michaelis constant (K_m), the lower the concentration

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of pyruvate required to give inhibition and therefore the lower the pyruvate concentration optimum. Since the pyruvate concentration optima were fairly broad, precise values for these could not be determined. The logarithms of the pyruvate concentration optima, however, were related approximately inversely to the electrophoretic mobilities. Two sets of conditions were selected, namely (1) pH 7.0 and 0.0025 *M* sodium pyruvate, which provided near optimum conditions for LDH₁ and inhibitory concentrations of pyruvate for the other isozymes, and (2) pH 8.0 and 0.0006 *M* sodium pyruvate, which provided near optimum conditions for LDH₅ and below-optimum pyruvate concentrations for the other isozymes. The ratio of the reaction velocities under these two sets of conditions, that is V_1/V_2 , permitted a precise comparison between the isozymes in regard to the factors determining the pyruvate concentration optima. As shown in FIGURE 1a the logarithms of these V_1/V_2 ratios are inversely proportional to the electrophoretic mobilities of the isozymes.

Heat stabilities. Plagemann *et al.*¹⁰ found marked differences in the heat stabilities of the electrophoretically purified rabbit LDH isozymes. The inactivation curves indicated a biphasic reaction. The declines in activity were preceded by latent periods, although the experiment was designed so that the isozymes were instantaneously brought to the inactivating temperature at zero time. After this latent period the decline in activity followed a first order reaction. The variation in heat stability of the isozymes was too great to enable velocity constants of inactivation to be determined for all five isozymes at any single temperature. These constants for the first order phase of the inactivation could be determined for LDH₁ to LDH₄ inclusive at 53° C. The logarithms of these velocity constants of inactivation are inversely proportional to the electrophoretic mobilities of the isozymes (FIGURE 1b).

Energy of activation. Plagemann *et al.*¹⁰ applied the Arrhenius equation for the calculation of apparent energies of activation (μ) values, for the conversion of lactate to pyruvate by the five rabbit LDH isozymes. In contrast to the reverse reaction, excess lactate was not inhibitive to the enzyme. Therefore concentrations of sodium lactate and diphosphopyridine nucleotide (DPN⁺), which were in excess of those required to allow near-maximum velocity of the reaction were employed for these determinations. It is not entirely clear which of the five individual reactions involved in the enzymatic conversion of lactate to pyruvate was rate-limiting under these conditions, although the studies of Winer and Schwert¹¹ suggest that it may have been the dissociation of the LDH-DPNH complex. The data showed marked differences between the apparent energy of activation values, these ranging from $8,285 \pm 400$ calories for LDH₁ to $13,188 \pm 74$ calories for LDH₅. These values, it was considered,¹⁰ show a linear correlation with the electrophoretic mobilities of the isozymes but with an irregular increment between LDH₁ and LDH₂. It is apparent from FIGURE 1c, however, that the data are equally compatible with a direct relationship between the logarithms of the " μ " values and the electrophoretic mobilities, without exceptions. In view of the fact that the logarithms of the other parameters described above correlated with the electrophoretic mobilities, FIGURE 1c is now considered to be better descriptive of the relationship between μ values and relative electrophoretic mobilities of the isozymes.

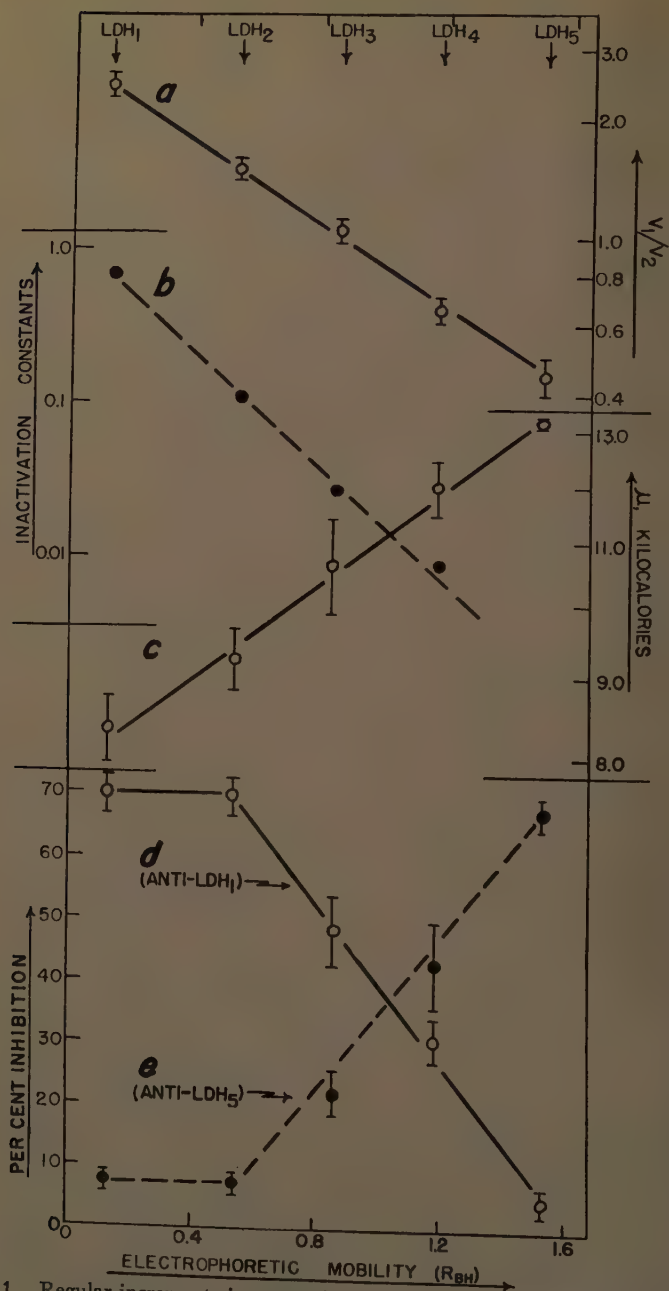


FIGURE 1. Regular increments in properties of rabbit LDH isozymes as correlated with their electrophoretic mobilities in starch gel relative to the migration front of bovine hemoglobin (R_{BH}). (a) Ratios of reaction velocities at pH 7.0 and 0.0025 *M* sodium pyruvate (V_1) and pH 8.0 and 0.0006 *M* sodium pyruvate (V_2). (b) Velocity constants of heat inactivation at 53° C. (c) Apparent energy of activation (μ) lactate to pyruvate, with excess sodium lactate and DPN⁺. (d) Per cent inhibition by antibody to LDH₁. (e) Per cent inhibition by antibody to LDH₅.

Since the ultimate function of an enzyme is frequently stated to be to "lower the energy of activation" of a reaction, the above data were first interpreted to mean that LDH₁ had the greatest catalytic efficiency of the five isozymes since it gave the lowest "apparent energy of activation".¹⁰ This conclusion seemed compatible with reports by Wieland *et al.*¹² to the effect that skeletal muscle LDHs (slow migrating forms) had turnover numbers appreciably higher than those for heart muscle LDHs (faster migrating forms). If the entropy of activation values for both LDH₁ and LDH₅ were the same, however and, if the apparent energy of activation values determined are accepted, then calculations based on the "theory of absolute reaction rates"¹³ show that, at 30° C., LDH₁ should have a turnover number approximately 3,300 times greater than LDH₅.

In order to investigate this point further Plagemann¹⁴ isolated LDH₅ from about 200 rabbit hearts (the rabbit heart contains only the LDH₅ isozyme). This enzyme was purified by a combination of the methods described by Straub¹⁵ and Wieland *et al.*¹² for the crystallization of beef heart LDH and rat heart LDH respectively. Turnover numbers of rabbit LDH₁ and LDH₅ at 30° C., pH 7.4, 1.5×10^{-4} M DPNH and pyruvate concentrations optimum for each isozyme, assuming a molecular weight of 100,000 for each LDH^{12,16} were found to be about 12,000 and 43,000 respectively. The ratios between the turnover numbers of these two isozymes is temperature-dependent since the temperature coefficients of the reaction as catalyzed by these two forms is quite different.¹⁰ It is apparent that the turnover numbers do not show the predicted disparity in values but are of the same order of magnitude.

As pointed out by Stearn¹⁷ it is a general rule that any catalytic modification of a reacting system that increases the value of the heat of activation (μ -RT) for a particular reaction also increases the value of the entropy of activation for the same reaction. The theory of absolute reaction rates stresses that the free energy of activation determines reaction rates. Since $\Delta F = \Delta H - T\Delta S$, where F = free energy of activation, ΔH = heat of activation, T = absolute temperature and ΔS = entropy of activation, it is apparent that increasing values of heat and entropy of activation have an opposite or compensating effect on the velocity of the reaction. Whereas the change in heat of activation is usually the dominant factor,¹⁷ it is apparent that, in the present instance, the change in entropy of activation values had as great an effect on the catalytic efficiency of the various isozymes as had the change in heat of activation. K. J. Laidler (University of Ottawa, Ont., Canada, personal communication) has suggested that in this system there may be relatively small differences between the isozymes that change the degree of binding of water molecules to the polar groups on the enzyme surface and in this way produce compensating changes in heat and entropy. Similar compensation has been noted previously in certain nonenzymatic reactions.¹⁸

Inhibition by antibody. An exception to the general finding of regular differences between the five rabbit LDH isozymes was encountered by use of the antibody formed against LDH₁ for the inhibition of the five isozymes.⁹ LDH₁ and LDH₂ were inhibited an identical amount by this antienzyme although the remaining isozymes were inhibited in regularly decreasing amounts which correlated with their electrophoretic mobilities (FIGURE 1d).

The serological specificity of these isozymes has been investigated further by means of antibody formed against purified LDH₅. The production and assay of anti-LDH₅ activity was as described previously for anti-LDH₁.¹ The relative degree of inhibition of each of the five LDH isozymes by anti-LDH₅ is shown in FIGURE 1e. It is apparent that the results were the exact inverse of the inhibitions of anti-LDH₁ (FIGURE 1d) and that once again LDH₁ and LDH₂ were serologically indistinguishable. When anti-LDH₁ was used at lower concentrations and when anti-LDH₅ was tested at higher concentrations, LDH₁ and LDH₂ were still inhibited to the same extent by each antienzyme.

LDH Isozymes in Rabbit Plasma

In view of the diagnostic implications of the distribution of LDH isozymes in human plasma,^{7,8} experiments were initiated on the experimental modification of isozyme proportions in rabbit plasma. The distribution of the isozymes

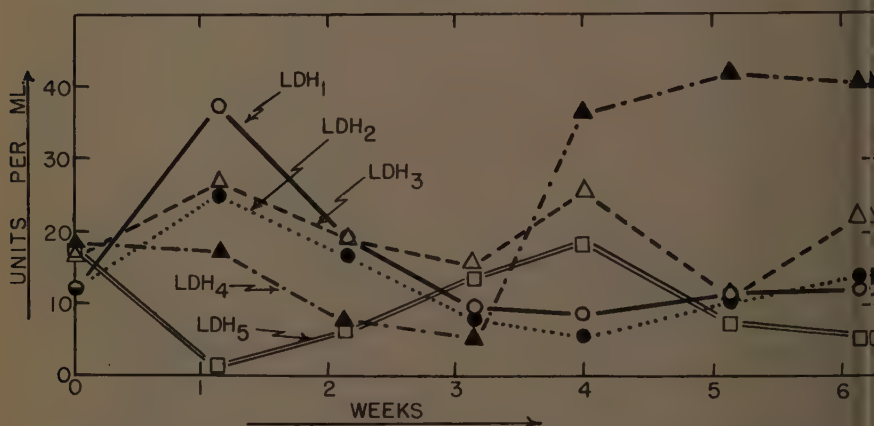


FIGURE 2. Changes in the LDH isozyme content of plasma from a normal rabbit.

zymes in plasma samples was routinely determined by subjecting plasma to starch gel electrophoresis as previously described⁴ and subsequently cutting the gel into 5 strips corresponding to the expected positions of the isozymes in relation to the migration front of the reference protein, bovine hemoglobin. Comparative studies showed reasonable agreement between this method and the results obtained by cutting and individually assaying 25 to 30 0.3-cm wide strips cut over the length of the gel. The total amount of each isozyme in the plasma was estimated from the proportion of each isozyme recovered compared to the sum of all the isozymes recovered, multiplied by the total LDH content of the plasma.

The changes occurring in the LDH isozyme pattern of the plasma of one of three untreated rabbits examined is shown in FIGURE 2. It is apparent that wide fluctuations occurred in both the total LDH activity and in the relative proportions of the isozymes. This result was in marked contrast to data obtained with normal human plasma where a comparatively uniform pattern was observed. Changes in the LDH isozyme patterns were also followed in

pairs of rabbits in which a carcinoma* had been transplanted intramuscularly, intraperitoneally, or into the lungs by means of intravenous injection of tumor cells. The results obtained from one of the rabbits bearing an intramuscular tumor are shown in FIGURE 3. The intramuscular tumors reached a detectable size by 3 weeks and, by the end of the experiment (6½ weeks), one of the rabbits with lung-tumors had died and all tumor-bearing animals were in an advanced state of emaciation. None of the animals studied showed a consistent pattern of isozyme distribution ascribable either to the site of tumor growth

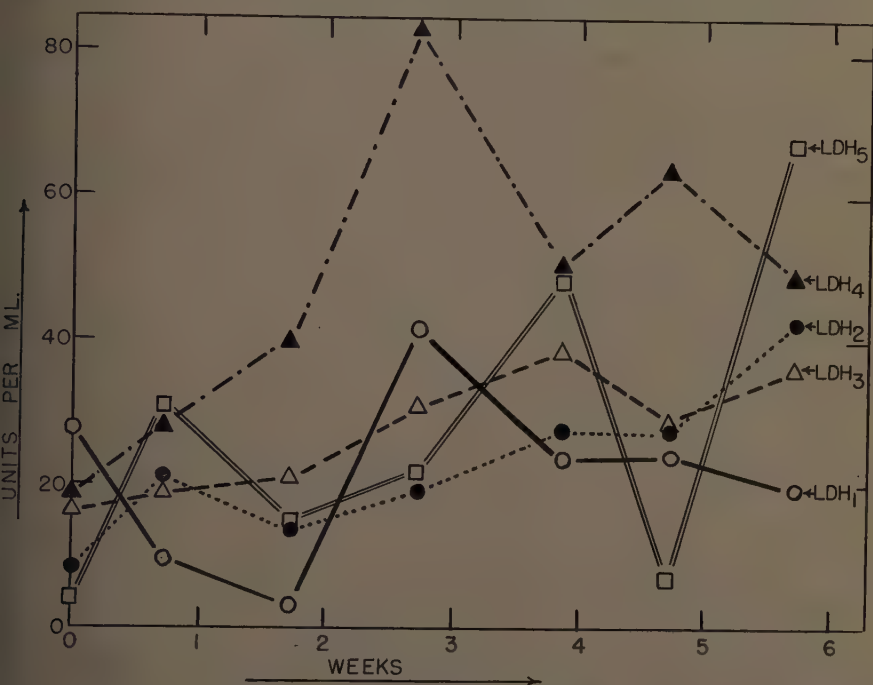


FIGURE 3. Changes in the LDH isozyme content of plasma from a rabbit bearing the Vx-7 tumor intramuscularly.

or to tumor growth *per se*. A slight elevation of total plasma LDH occurred in the intramuscular tumor-bearing rabbits, but this elevation did not result from an increase in the amount of any particular isozyme. The animals bearing tumors at the other sites showed no elevation in total LDH. In all cases the fluctuation in the proportion of the 5 isozymes was even greater in the plasma of tumor-bearing rabbits than it was in the plasma of the control rabbits.

Studies with human plasma from certain disease conditions had indicated that when one isozyme had been elevated (for example, LDH₅ following myocardial infarction) then the isozyme pattern remained abnormal for some time even after the total plasma LDH had returned to normal levels. The previ-

* Vx-7, originally selected by Peyton Rous and in its 62nd transplant generation.

ously nonelevated isozymes were, therefore, actually present in decreased concentrations. These data suggested that a homeostatic mechanism in the circulating system might act indiscriminately on the 5 isozymes while lowering total LDH activity to normal levels. This possibility was explored in the rabbit by injecting approximately 1.0×10^6 units of commercial* rabbit muscle LDH (LDH₁) into pairs of rabbits intravenously, subcutaneously, and intraperitoneally. Rabbits given LDH intravenously showed an immediate elevation of plasma LDH from a normal level of about 100 U./ml. to several thousand U./ml. The level rapidly decreased with time, usually approaching normal levels 6 to 8 hours after injection. Rabbits given subcutaneous and intraperitoneal injections of LDH₁ showed about a threefold increase in total plasma LDH level in about 1½ hours, following which the levels slowly returned to normal in 18 to 24 hours.

In the present experiment blood samples were drawn into heparin about one half hour before the injection of LDH₁ and again at a time when total LDH

TABLE 1
SELECTIVE HOMEOSTATIC ACTION IN THE RABBIT ON LDH₁ IN PLASMA

Route of injection of LDH ₁ *	Time of sampling (hours)	Units per milliliter plasma of LDH number:				
		1	2	3	4	5
Intravenous	-0.5	25.6	35.0	54.7	146.5	52.1
	+4.0	150.2	68.2	71.5	101.0	72.1
Subcutaneous	-0.5	12.0	12.0	22.3	49.0	3.8
	+18.0	20.4	15.1	17.0	33.1	4.8
Intraperitoneal	-0.5	13.0	9.1	19.5	29.0	18.2
	+18.0	9.1	5.5	16.3	26.6	5.5

* One $\times 10^6$ U. of commercial rabbit muscle LDH (LDH₁) injected at $t = 0$.

had just returned or nearly returned to normal levels (4 hours after intravenous injections, 18 hours after subcutaneous and intraperitoneal injections). If the mechanism responsible for the rapid elimination of this large excess of total LDH from the plasma was unable to discriminate between the various isozymes, then all isozymes should decrease in concentration at the same proportional rate. Therefore, by the time that total LDH had been lowered to approximately normal levels, the concentrations of LDH₂ to LDH₅ inclusive in the plasma should have been markedly lower than prior to the injection of the LDH₁. The data clearly indicated that this did not occur (TABLE 1) but that this mechanism acted selectively on the added LDH₁. It is evident, however, that this "homeostatic mechanism" is markedly ineffective in maintaining uniform levels of the LDH isozymes in normal rabbit plasma (FIGURE 2).

The contrary data obtained with human plasma occurred in instances where diseased or damaged tissues were presumably leaking LDH into the blood stream over a period of several days. The apparent depression of the level

* Nutritional Biochemical Corporation, Cleveland, Ohio.

of the LDH isozymes not coming from the injured tissue may mean that the normal replenishment of plasma LDH from various tissues was nonselectively repressed by the elevated total plasma LDH. On the other hand the differences may result from a difference in the degree of selectivity of the mechanism responsible for LDH elimination in these two species.

Heat Stability of Human LDH Isozymes in Serum

Although changes occurring in the proportion of LDH isozymes in serum has proved diagnostic value^{7,8} the technical requirements of the electrophoretic procedure militate against the widespread adoption of this technique for routine diagnosis. The marked differences observed in the heat stabilities of the rabbit LDH isozymes¹⁰ (FIGURE 1b) suggested that, if the LDH isozymes in

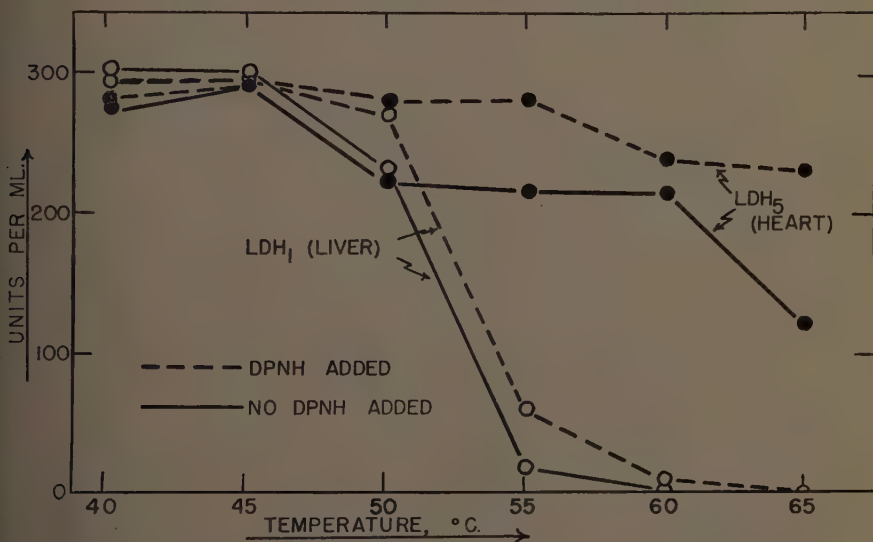


FIGURE 4. Amount of added human LDH₁ and LDH₅ in serum remaining after treatment for 30 min. at various temperatures.

human serum showed similar variations in heat stabilities, it might be possible to exploit these differences in simple diagnostic tests. LDH₁ and LDH₅ were purified by starch gel electrophoresis from liver and heart tissue, respectively, obtained from an autopsy performed four hours after death.* To samples of pooled normal serum of relatively low LDH content (120 U./ml.) was added sufficient of one or the other of these isozymes to give final total LDH concentrations of ca. 410 U./ml. One half of each type of supplemented serum and a portion of nonsupplemented serum was supplemented with DPNH (0.25 mg./ml.). These mixtures were allowed to stand overnight at 4° C. The variously supplemented serum and normal serum samples were dispensed in 0.5 ml. amounts in small test tubes (inside diameter = 8 mm.) and immersed in mechanically stirred constant temperature water baths adjusted at 5° increments from 40° C. to 70° C. inclusive. The tubes were removed after

* Kindly supplied by E. L. Barton, General Hospital, Guelph, Ont., Canada.

exactly 30 min. immersion in the water baths, placed in an ice bath, and subsequently assayed for total remaining LDH activity. The 70° C. treated samples coagulated and could not be assayed. The total LDH in an isozyme-supplemented sample minus the total LDH in the corresponding nonsupple-

TABLE 2
ELECTROPHORETIC MIGRATION DISTANCE OF HUMAN LACTIC DEHYDROGENASE
RELATIVE TO THE MIGRATION FRONT OF PURIFIED BOVINE HEMOGLOBIN

LDH	
1	-0.21 \pm 0.02
2	0.39 \pm 0.03
3	0.75 \pm 0.02
4	1.14 \pm 0.05
5	1.53 \pm 0.05

TABLE 3
PERCENTAGE COMPOSITION OF THE ISOZYMES OBSERVED ELECTROPHORETICALLY
OF PLASMA OF NORMAL ADULT INDIVIDUALS

	LD ₁	LD ₂	LD ₃	LD ₄	LD ₅
Percentage*	0-5	0-15	0-35	25-50	10-35

* Range includes twice the standard deviation from mean value.

B.A., 50, ♂. Normal adult

Total plasma LD = 300 units/ml.

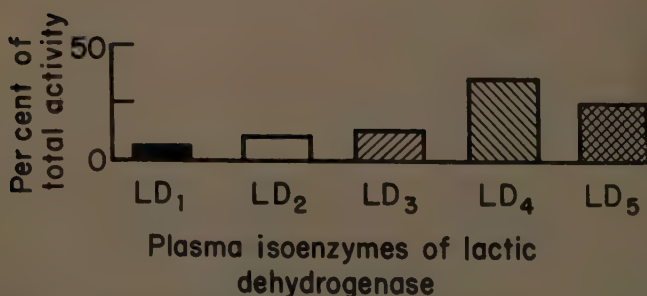
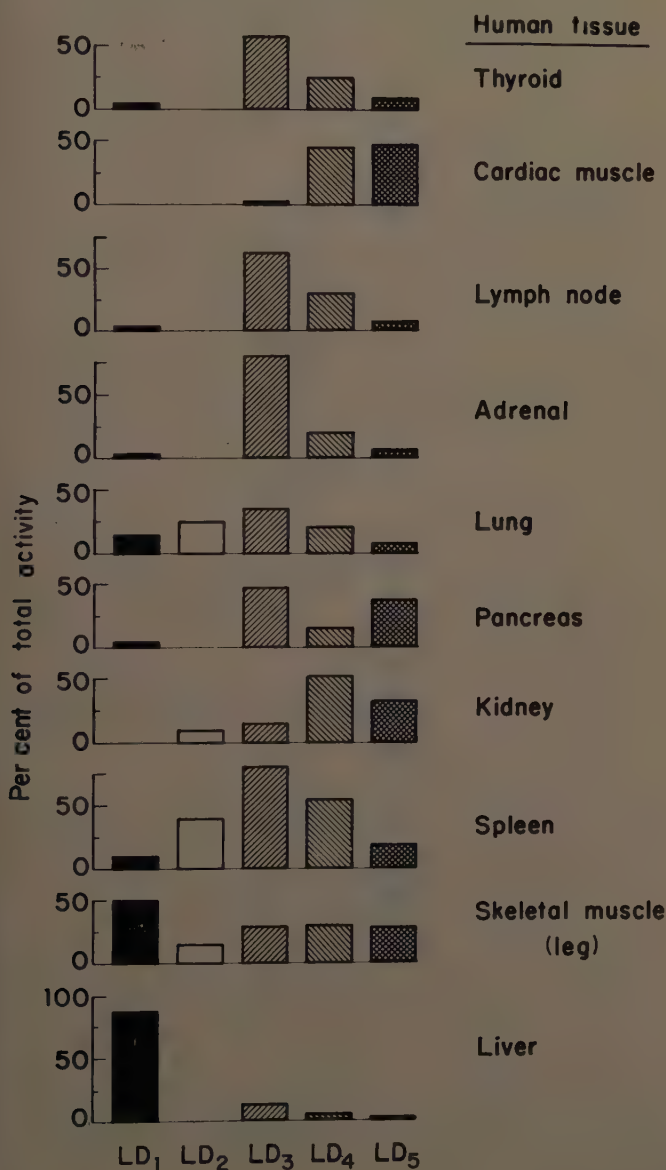


FIGURE 5. LDH isozyme composition of plasma of normal adult individual.

mented sample gave the amount of added LDH isozyme remaining after the treatment. The results (FIGURE 4) indicated a marked difference between the heat stabilities of LDH₁ and LDH₅ in serum. Repetition of the experiment at the critical temperatures of 55° C. and 65° C. yielded very similar data. DPNH increased the heat stability of both isozymes. The results obtained with LDH₅ suggested the possibility that this electrophoretically purified iso-

zyme might in fact be composed of 2 components that differed in heat stability since, in the absence of added DPNH, treatment at 50° C. destroyed part of the enzymatic activity but further enzyme was not destroyed until a temperature of 65° C. was reached. The presence of added DPNH, however, increased



Plasma isoenzymes of lactic dehydrogenase

FIGURE 6. LDH isozyme composition of watery extracts of normal human tissues.

the stability of this putative heat-labile component much more than it increased the stability of the similarly labile LDH₁.

These results are of a preliminary nature but strongly suggest that a simple differential test based on the relative heat stabilities of the human LDH isozymes in serum is possible. Such a test could readily be used in a routine

F. R., 49 yr., ♂. Anteroseptal myocardial infarction

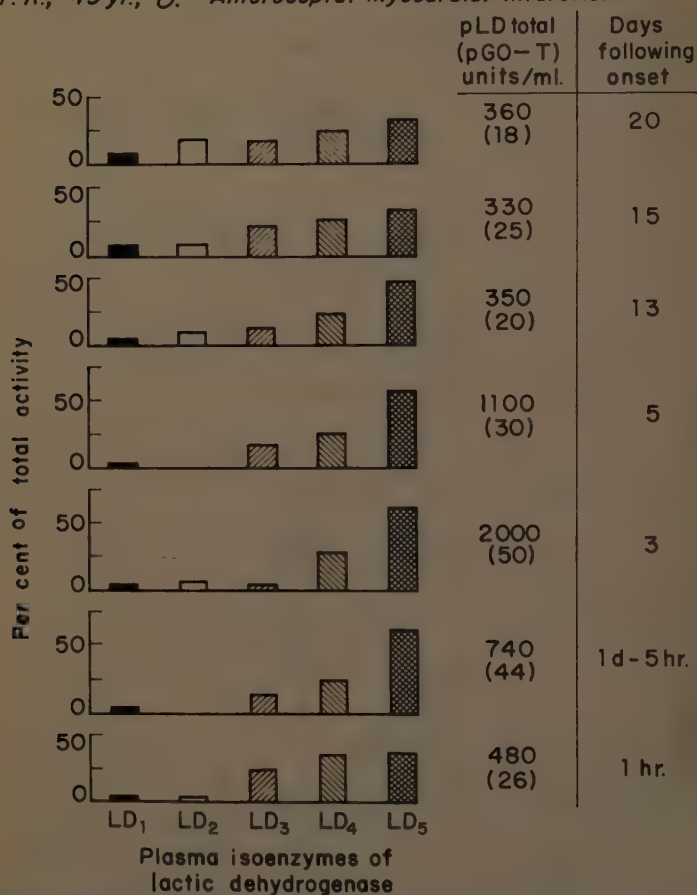


FIGURE 7. Serial LDH isozyme composition of plasma of patient who incurred anteroseptal myocardial infarction.

way in any clinical laboratory. The following procedure is tentatively suggested:

(1) To 2 ml. of unhemolyzed serum add 0.2 ml. DPNH solution (2.5 mg./ml.). (Plasma cannot be used as the fibrinogen coagulates at the higher temperature used.)

(2) Mix and let stand 20 min.

(3) Place 0.5 ml. amounts of the serum into each of 3 small test tubes.

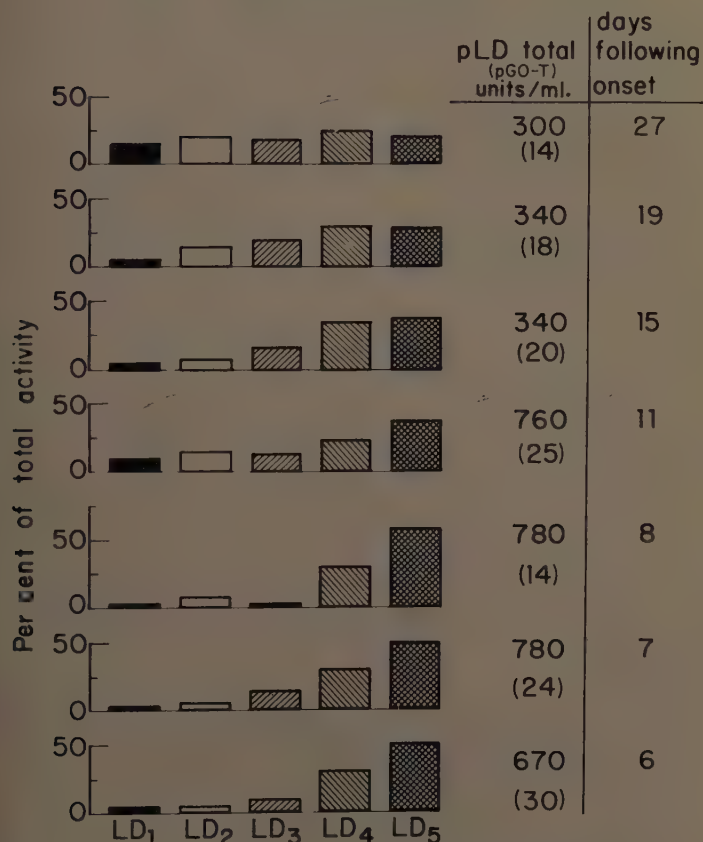
(4) Leave one tube unheated and place the other 2 tubes in mechanically stirred water baths at 57° C. and 65° C. for exactly 30 min.

(5) Remove the tubes, cool rapidly, and determine LDH activity in all 3 portions as described by Wróblewski and LaDue.²⁰

The results are interpreted as follows:

- (1) Unheated sample = total LDH.
- (2) Unheated sample - 57° C. heated sample = heat labile LDH (mostly LDH₁ ; elevated in hepatic diseases).

J.W. 49 yr., ♂. Posterior myocardial infarction



Plasma isoenzymes of lactic dehydrogenase

FIGURE 8. Serial LDH isozyme composition of plasma of patient who incurred posterior myocardial infarction.

(3) Heated sample (65° C.) = heat stable LDH (mostly LDH₅ ; elevated in myocardial infarction).

(4) Heated sample (57° C.) - 65° C. heated sample = LDHs of intermediate heat stability.

Isozymes of Normal Human Tissue Homogenates and Plasma

Heparinized plasma obtained from normal adults without regard to the fasting state had a total lactic dehydrogenase of 360 to 60 U. per milliliter,

with a range of activity from 240 to 480 U. at 30° C. The plasma of normal adults usually contained all 5 plasma isozymes (pLD₁, pLD₂, pLD₃, pLD₄, and pLD₅). In several subjects, however, either pLD₁ or pLD₂ were not present. TABLE 2 lists the electrophoretic migration of human lactic dehydrogenase relative to the migration front of purified bovine hemoglobin (R_{BHb}). TABLE 3 lists the approximate percentage composition of the isozyme observed.

*I. G., 70, ♂. Subendocardial infarction
(secondary to shock)*

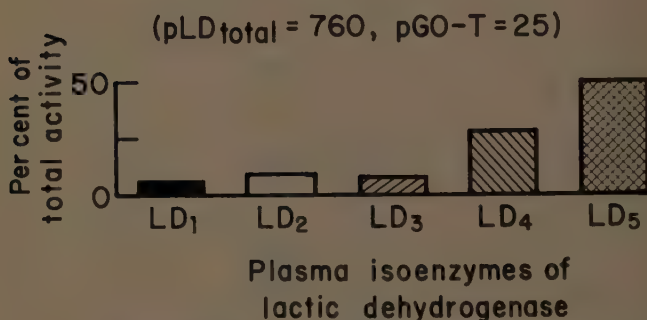


FIGURE 9. LDH isozyme composition of plasma of patient who incurred subendocardial infarction during state of shock.

J. K., 38 yr., ♀. Acute thyroiditis

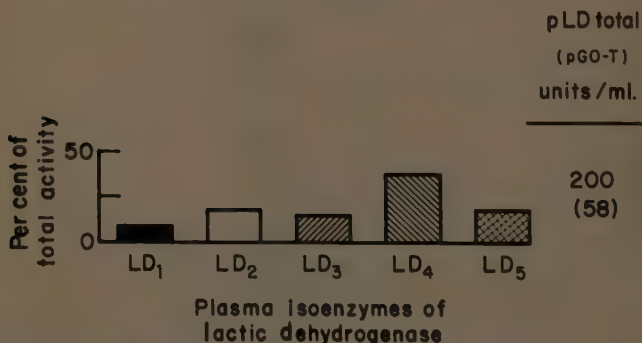


FIGURE 10. LDH isozyme composition of plasma of patient with acute thyroiditis.

by the electrophoresis of plasma of normal adults, and FIGURE 5 depicts the plasma isozyme pattern of one such person. In all cases pLD₄ was present in amounts greater than pLD₅.

Examination of the lactic dehydrogenase isozymes of normal human tissues indicates that different tissues contain different numbers of the five isozymes and in distinct patterns. FIGURE 6 presents the isozyme composition and pattern of human heart muscle, skeletal muscle, liver, kidney, spleen, lung, and others.

Alteration of Isozymes of Plasma in Patients with Various Disease States

Plasma obtained from patients with myocardial infarction showed an increased content of pLD₄ and pLD₅, with the predominant increase in the latter. FIGURE 7 presents the plasma isozyme patterns observed during the clinical course of a patient who incurred an anteroseptal myocardial infarction. Plasma isozyme studies from another patient with myocardial infarction from

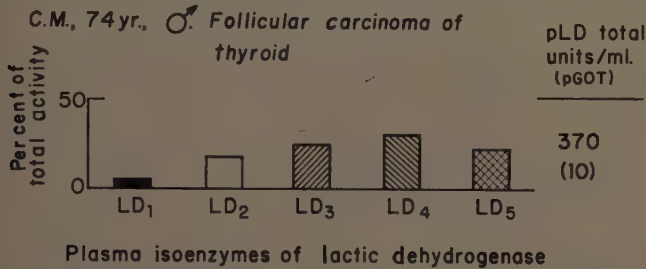


FIGURE 11. LDH isozyme composition of plasma of patient with follicular carcinoma of thyroid.

K.V., 56 yr., ♀ Infectious hepatitis

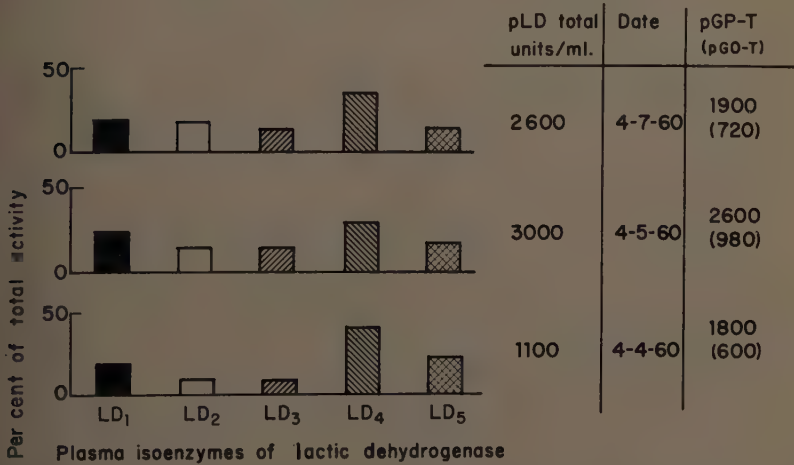


FIGURE 12. Serial LDH isozyme composition of plasma of patient with infectious hepatitis.

the 6th to the 27th day showed an increased percentage of pLD₅, which was consistently greater than pLD₄ (FIGURE 8). The peak pLD₅ was greater than the peak pLD₄, and there was a relative and, at times, an absolute decrease in the three other isozymes at the time that pLD₅ was maximal. FIGURE 9 shows the plasma isozyme pattern seen during the clinical course of a patient with subendocardial infarction. Although total plasma LD (pLD_t) was slightly elevated (while plasma glutamic oxaloacetic transaminase remained normal), the alteration could not be related to myocardial necrosis specifically,

since other circumstances surrounding the shocklike clinical picture could have accounted for the elevated pLD_t. The plasma isozyme pattern showed an appreciable increase in pLD₄ and pLD₅, the latter being greater than the former. These isozyme changes persisted for 10 days. Although in extra-

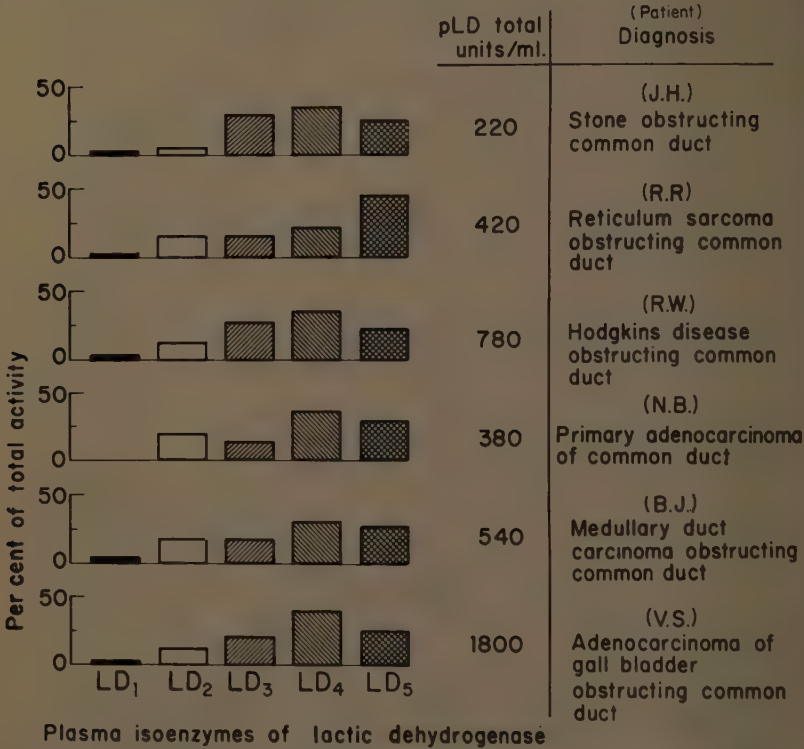


FIGURE 13. LDH isozyme composition of the plasma of a group of patients with common duct obstruction due to various etiologic factors.

I. McA., 55 yr., ♂. Pulmonary infarction

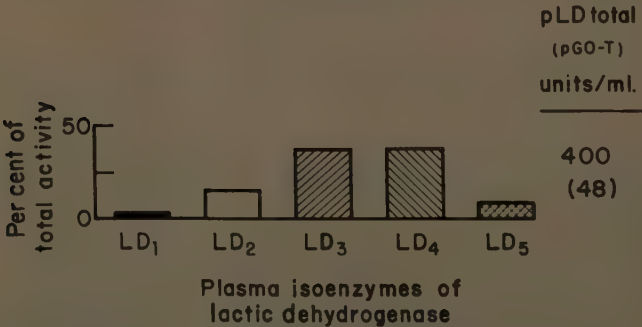


FIGURE 14. LDH isozyme composition of plasma of a patient who incurred a pulmonary infarction.

cardiac disease pLD_t alterations may be quantitatively and, at times, serially similar to those observed in myocardial infarction, the plasma isozymes are distinctly dissimilar.

FIGURES 10 to 19 depict isozyme patterns observed when the plasma of patients with various diseases were examined. It appears that the isozyme components of the tissue or organ diseased are contributed to the plasma and are in part added to the isozyme components normally found in the plasma. Homeostatic factors influence LD activity of the plasma and result in variations from the hypothetically expected isozyme patterns of plasma when the isozyme

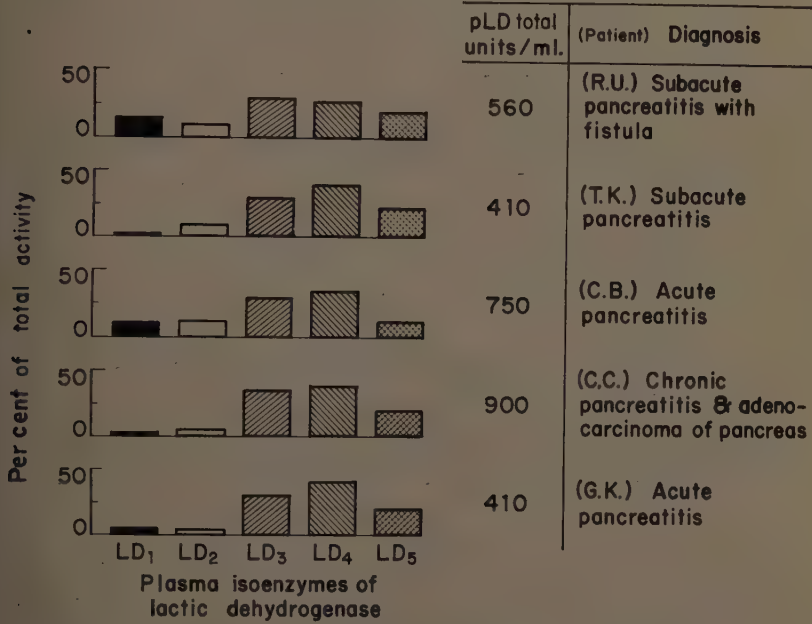
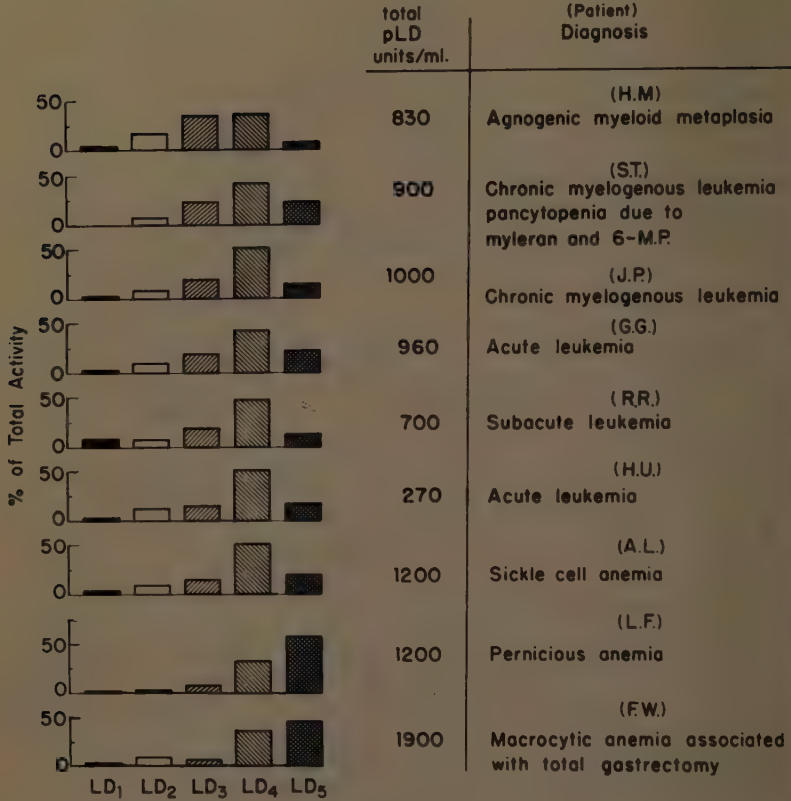


FIGURE 15. LDH isozyme composition of the plasma of a group of patients with pancreatic disease.

component of the diseased tissue are added to the components usually present in the plasma.

Serum Isozyme Alterations Determined by Relative Heat Stability Test

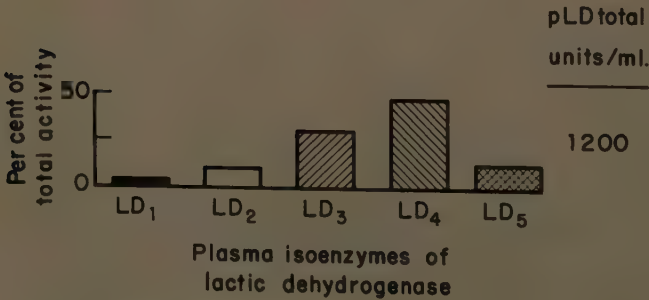
Using the simple relative heat stability test, the serum of normal adult individuals, and the serum of patients with various diseases were studied for LDH₁, LDH_{2,3,4}, and LDH₅. TABLE 4 presents the total serum LDH activity and the percentage composition of LDH₁, LDH_{2,3,4}, and LDH₅ in normal adult individuals. TABLE 5 lists the total serum LDH and percentage composition of LDH₁, LDH_{2,3,4}, and LDH₅ obtained in a group of patients with various disease states. The simple relative heat stability test permits a rapid estimate of change in composition of component LDH₁, LDH₅, and the serum of LDH₂, LDH₃, and LDH₄. The data obtained by heat stability test differ from the estimates derived from starch-gel electrophoretic separa-



Plasma isoenzymes of lactic dehydrogenase

FIGURE 16. LDH isozyme composition of the plasma of a group of patients with various hematologic disease states.

E.S., 35 yr., ♀. Adenocarcinoma of adrenal cortex



Plasma isoenzymes of
lactic dehydrogenase

FIGURE 17. LDH isozyme composition of the plasma of a patient with adrenal cortical carcinoma.

N.K., 18 yr, ♀. Acute glomerulonephritis

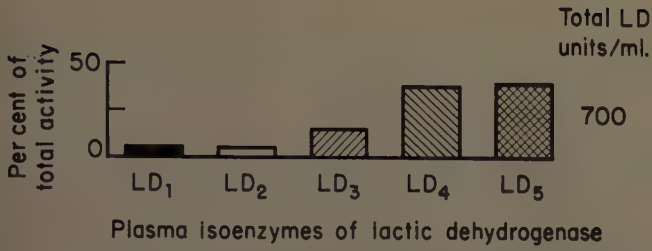


FIGURE 18. LDH isozyme composition of the plasma of a patient with acute glomerulonephritis.

L.F. 31, ♀. Pernicious anemia

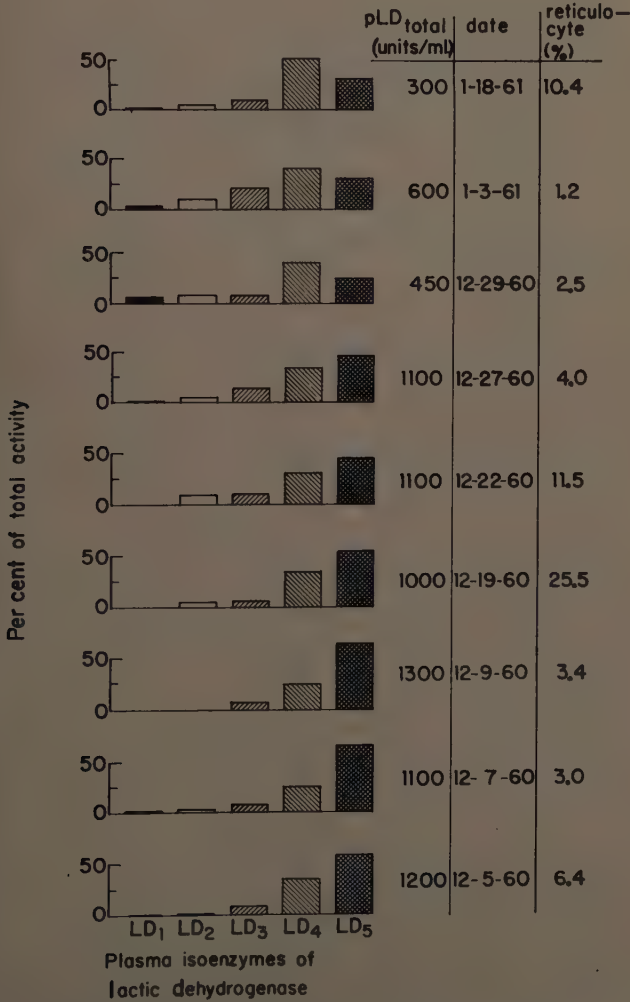


FIGURE 19. Serial LDH isozyme composition of the plasma of a patient with pernicious anemia who was treated with vitamin B₁₂ starting on December 9, 1960.

tion. The larger values obtained from LDH₁ presumably reflect the loss of LDH₁ during electrophoresis because of inactivation due to heat generated by the electrical flow. It appears that for rapid and simple estimation of LDH₁

TABLE 4
PERCENTAGE COMPOSITION OF THE ISOZYMES OBTAINED BY HEAT STABILITY
TEST OF PLASMA OF NORMAL ADULT INDIVIDUALS

Serum LDH component	Range of composition in percentage* of total
LDH ₁	10-30
LDH _{2,3,4}	35-55
LDH ₅	20-40

* Range includes twice the standard deviation from mean value.

TABLE 5
TOTAL SERUM LDH ACTIVITY AND PERCENTAGE COMPOSITION OF THE ISOZYMES OBTAINED
BY HEAT-STABILITY TEST OF THE PLASMA OF PATIENTS WITH VARIOUS
DISEASE STATES

Patient	Diagnosis	Total serum LDH (U./ml.)	Serum LDH ₁ (%)	Serum LDH _{2,3,4} (%)	Serum LDH ₅ (%)
J.D.	Myocardial infarction	680	12	38	50
J.E.	Myocardial infarction	1800	15	29	56
G.G.	Myocardial infarction	1200	12	33	45
A.L.	Myocardial infarction	2000	10	30	60
N.De	Infectious mononucleosis hepatitis	240	33	49	18
G.U.	Viral hepatitis	390	37	43	20
F.M.	Homologous serum hepatitis	250	43	43	14
T.P.	Homologous serum hepatitis	360	33	49	18
M.J.	Homologous serum hepatitis	400	32	53	15
M.R.	Homologous serum hepatitis	2100	85	10	5
T.R.	Homologous serum hepatitis	1700	77	15	8
G.M.	Metastatic melanoma to liver	310	36	38	26
D.B.	Metastatic carcinoma to liver	620	40	29	31
F.H.	Multiple myeloma	270	20	44	36
S.Y.	Gastric carcinoma	2400	0	54	46
E.M.	Carcinoma of thyroid	620	27	56	17
S.L.	Rectal carcinoma with liver and pulmonary metastasis	1500	42	43	15
S.T.	Gastric carcinoma	5500	17	51	32
C.K.	Acute glomerulo nephritis	720	17	50	33
B.B.	Emphysema	290	27	55	18
L.F.	Pernicious anemia (remission)	260	17	49	34
E.F.	Lupus erythematosus	470	24	42	34
J.D.	Bronchopneumonia	600	38	40	22

and LDH₅, as well as the serum of LDH₂, LDH₃, and LDH₄, the relative heat stability test is satisfactory.

Summary

Five distinct forms (isozymes) of lactic dehydrogenase were separated from both rabbit and human tissues and plasma by zone electrophoresis in starch gel. The five forms from the rabbit showed regular increments in the follow-

ing four parameters that correlated with the electrophoretic mobilities of the isozymes: (1) log. of the ratios of reaction velocities when tested under two sets of conditions of *pH* and pyruvate concentration that were optimum for the isozymes of the electrophoretic extremes; (2) per cent inhibition by antibody produced against the slowest migrating form (LDH₁) and by antibodies produced against the fastest migrating form (LDH₅) with the exception, in both cases, of identical inhibition of LDH₁ and LDH₂; (3) log. of the velocity constant of heat inactivation (53° C.); (4) log. of the apparent energy of activation (lactate to pyruvate).

Assays of the isozyme composition of homogenates of normal human tissues indicated that each tissue contains from one to five isozymes in characteristic proportions. No two human tissues examined appeared to be exactly the same in LDH isozyme composition. The plasmas from normal adult individuals contain four or five isozymes in a characteristic pattern. Clinical studies indicate that when a tissue is diseased or damaged it contributes those isozymes contained therein, resulting in a shift of the plasma isozyme pattern from the normal towards an abnormal pattern characterized by an increase in the isozymes present in the affected tissue. The distribution of isozymes in human plasma has diagnostic implications, especially for diseases of the heart muscle, skeletal muscle, liver, kidney, thyroid, and pancreas.

A tentative simple test for the determination of LDH₁ and LDH₅ in human serum, based on the differential heat stability of the isozymes, is described.

References

1. FUTTERMAN, S. & J. H. KINOSHITA. 1959. Metabolism of the retina. II. Heterogeneity and properties of the lactic dehydrogenase of cattle retina. *J. Biol. Chem.* **234**: 3174-3178.
2. HESS, B. & S. WALTER. 1960. Über das Protein der Laktatdehydrogenase im menschlichen Serum und Geweben. *Klin. Wochensh.* **38**: 1080-1088.
3. MARKERT, C. L. & F. MØLLER. 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci. U.S.A.* **45**: 753-763.
4. PLAGEMANN, P. G. W., K. F. GREGORY & F. WRÓBLEWSKI. 1960. The electrophoretically distinct forms of mammalian lactic dehydrogenase. I. Distribution of lactic dehydrogenases in rabbit and human tissues. *J. Biol. Chem.* **235**: 2282-2287.
5. VESELL, E. S. & A. G. BEARN. 1958. The heterogeneity of lactic and malic dehydrogenase. *Ann. N.Y. Acad. Sci.* **75**(1): 286-291.
6. WIELAND, T., G. PFLEIDERER, I. HAUPT & W. WÖRNER. 1959. Über die Verschiedenheit der Milchsäuredehydrogenasen. IV. Quantitative Ermittlung einiger Enzymverteilungsmuster Vergleichende Betrachtung bei verschiedenen Wirbeltierklassen. *Biochem. Z.* **332**: 1-10.
7. WIEME, R. J. 1959. Studies on Agar Gel Electrophoresis. *Arsicia Uitgaven N.V.* Brussels, Belgium.
8. WRÓBLEWSKI, F., C. ROSS & K. GREGORY. 1960. Isoenzymes and myocardial infarction. *New Engl. J. Med.* **263**: 531-536.
9. PLAGEMANN, P. G. W., K. F. GREGORY & F. WRÓBLEWSKI. 1960. The electrophoretically distinct forms of mammalian lactic dehydrogenase II. Properties and interrelationships of rabbit and human lactic dehydrogenase isozymes. *J. Biol. Chem.* **235**: 2288-2293.
10. PLAGEMANN, P. G. W., K. F. GREGORY & F. WRÓBLEWSKI. 1961. Die elektrophoretisch trennbaren Laktat-dehydrogenasen des Säugetieres III. Einfluss der Temperatur auf die Laktatdehydrogenasen des Kaninchens. *Biochem. Z.* **334**: In press.
11. WINER, A. D. & G. W. SCHWERT. 1958. Lactic dehydrogenase IV. The influence of *pH* on the kinetics of the reaction. *J. Biol. Chem.* **231**: 1065-1083.
12. WIELAND, T., G. PFLEIDERER & F. ORTANDERL. 1959. Über die Verschiedenheit der Milchsäuredehydrogenasen. *Biochem. Z.* **331**: 103-109.
13. LAIDLER, K. J. 1958. *The Chemical Kinetics of Enzyme Action.* Clarendon Press. Oxford, England.

14. PLAGEMANN, P. G. W. 1960. A study of the lactic dehydrogenase isozymes of rabbit and human tissues and their inhibition of specific antibody. M.S.A. Thesis. Univ. of Toronto, Toronto, Ont., Canada.
15. STRAUB, F. B. 1940. Crystalline lactic dehydrogenase from heart muscle. *Biochem. J.* **34**: 483-486.
16. PFLEIDERER, G. & D. JECKEL. 1957. Individuelle Milchsäuredehydrogenasen bei verschiedenen Säugetieren. *Biochem. Z.* **329**: 370-380.
17. STEARN, A. E. 1949. Kinetics of biological reactions with special reference to enzymic processes. *Advances in Enzymol.* **9**: 25-74.
18. LAIDLER, K. J. 1959. Thermodynamics of ionization processes in aqueous solution. *Trans. Faraday Soc.* **55**: 1725-1730.
19. GREGORY, K. F. & F. WRÓBLEWSKI. 1958. Preparation and properties of purified antilactic dehydrogenase. *J. Immunol.* **81**: 359-367.
20. WRÓBLEWSKI, F. & J. S. LADUE. 1955. Lactic dehydrogenase activity in blood. *Proc. Soc. Exptl. Biol. Med.* **90**: 210-213.

ISOENZYMES IN THE HUMAN EPIDERMIS

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This account of our examination of different molecular forms of lactic dehydrogenase, which we present as a preliminary report, does not serve the purpose of finding new diagnostic possibilities in dermatoses, but it has the aim of answering open and frequently problematic questions on the enzyme metabolism of the human skin. Substantially four problems are concerned and, because of the different character of each, they can be treated only independently.

(1) Are the enzymes and enzyme systems found in the human epidermis produced by the cells of the epidermis themselves or do they diffuse from the blood vessels of the dermis into these cells?

Weber and his co-workers could demonstrate¹⁻⁸ by extensive bioenzymatical investigation that different metabolites and enzymes of the glycolysis—among them also the lactic dehydrogenase—are found in high concentration in the cells of the epidermis, and that these diminish continuously in the direction of the peripheral bloodstream (FIGURE 1). From this decrease of concentration Weber and his associates inferred that the enrichment of enzymes in the epidermis is caused by the production of enzymes in the cells of the epidermis, and not by diffusion from the bloodstream, which would tend to accentuate the concentration decrease. Although these results represent convincing arguments, definite proof for the correctness of this thesis was still lacking.

This proof could be obtained by an electrophoretic determination of lactic dehydrogenase in a homogenate of the epidermis, of the dermis, and of erythrocytes which, according to Hess,¹⁴ contain the main portion of serum lactic dehydrogenase. As to method, we have followed Wieland *et al.*¹² As shown in FIGURE 2, there are such significant differences between the distribution patterns of lactic dehydrogenase in the epidermis and in the erythrocytes that from this fact alone the autochthonous production of lactic dehydrogenase in the cells of epidermis can be assumed with certainty.

(2) Is there any difference between the molecular forms of lactic dehydrogenase in the epidermis and in the dermis?

It would be necessary first to find if the concentration of lactic dehydrogenase in the epidermis is much higher than in the dermis. Comparing the distribution patterns of this enzyme in both tissues (FIGURE 2), a distinct shift to the right results for the epidermis, that is, column 5 is accentuated, whereas the distribution pattern of enzyme in the dermis shows a shift to the left, that is, a more distinct accentuation to column 1. According to Pfeleiderer and Wachsmuth¹³ the strictly anaerobic metabolizing tissues show a shift to the right, that is, the maxima are directed to column 5, whereas the tissues that have a high oxygen consumption are characterized by a shift to the left: that is, by maxima directed to column 1. The conclusion is that, in the case of cells of the epidermis, chiefly anaerobic metabolizing tissue is to be assumed: in the case of the cells of the dermis, a more aerobic metabolizing tissue is indicated.

(3) What origin have the enzymes in the case of pathological keratinisation in scales of the epidermis?

Weber^{5,8-11} showed, in comparing determinations of enzymes in orthokeratotic, hyperkeratotic, and parakeratotic horny layers, that frequently a considerable enrichment of enzymes in scales of the epidermis must be assumed in a pathological increased keratinization takes place. This holds true especially in the parakeratotic keratinization of psoriasis vulgaris. The pathohisto-

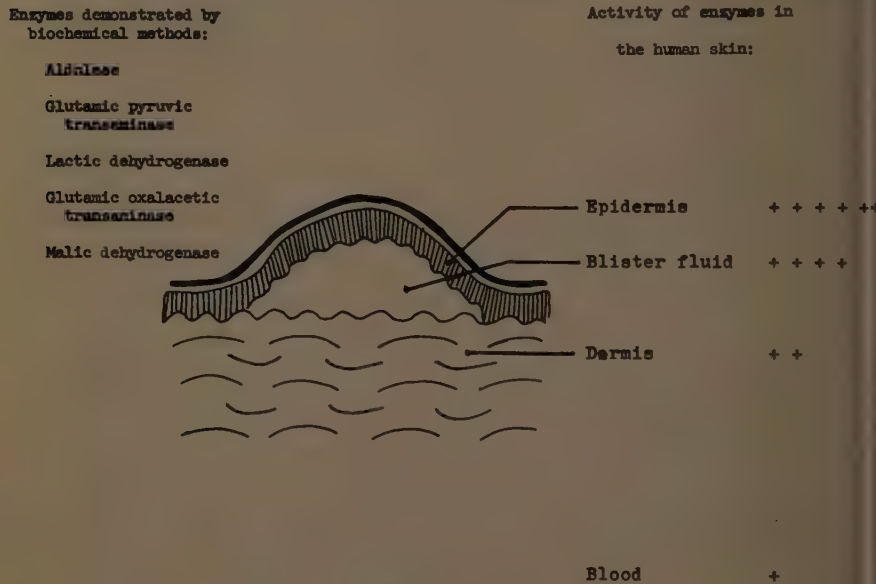


FIGURE 1.

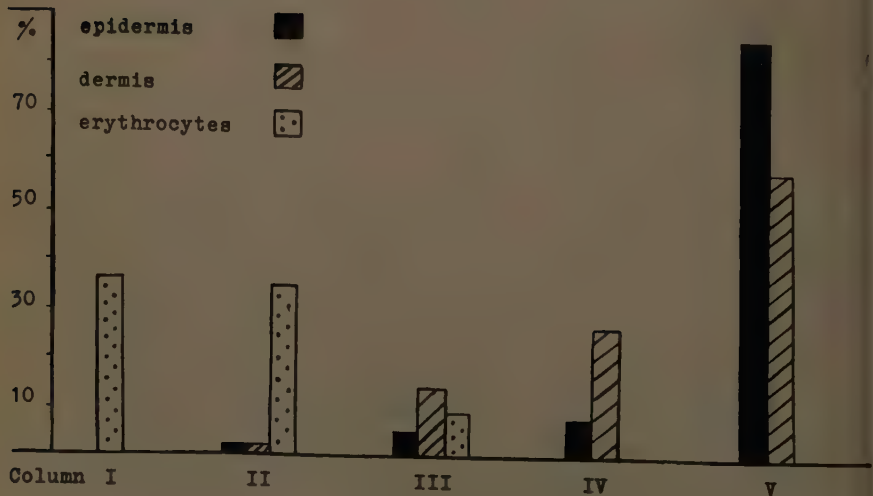


FIGURE 2.

logically demonstrable dilatation of the capillaries, as well as the accumulation of leukocytes well known as Munro microabscesses located in the stratum corneum or directly beneath it, suggest the possibility that the enzymes found in scales of the epidermis by Weber⁹⁻¹¹ are of secondary origin. This means

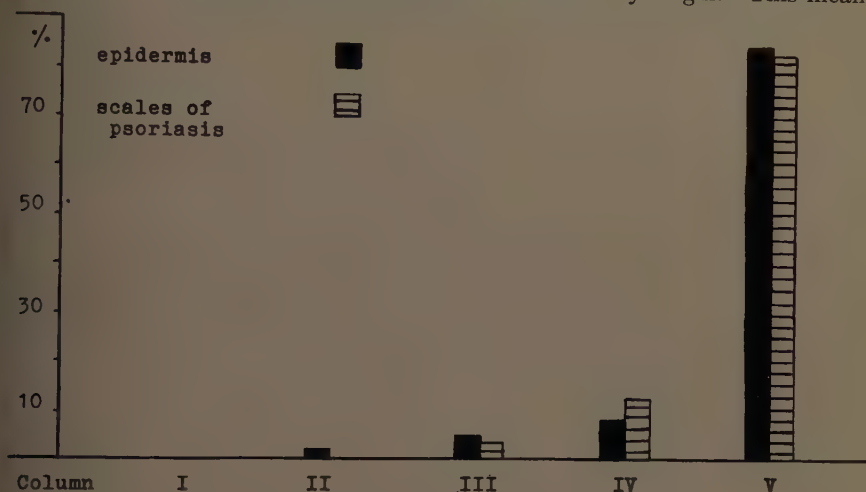


FIGURE 3.

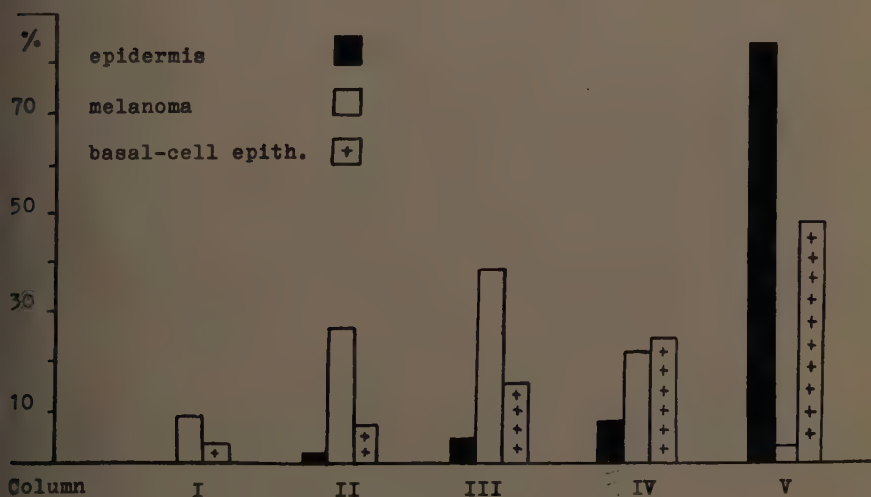


FIGURE 4.

that the enzymes come from the blood stream or from the immigrated leukocytes. The enzyme distribution pattern of the lactic dehydrogenase from scales of psoriasis vulgaris shown in FIGURE 3 demonstrates without any doubt that its molecular form is identical with that of the cells of the epidermis.

(4) Is the distribution pattern of the lactic dehydrogenase of basal-cell epithelioma and of malignant melanoma identical with that of the epidermis?

Thus far our preliminary examinations do not justify a definite explanation but they show, in the case of malignant melanoma, a heavily diminished column 5 and more pronounced columns 2, 3, and 4 (FIGURE 4). In the case of basal-cell epithelioma, column 5 is more pronounced and columns 2, 3, and 4 less so. This indicates clearly that neither the basal-cell epithelioma nor the malignant melanoma is identical with the epidermis with regard to the distribution pattern of lactic dehydrogenase.

References

1. WEBER, G. 1958. Über das Verhalten der Glutaminsäure-Oxallessigsäure-Transaminase im Blutserum und der Cantharidenblasenflüssigkeit bei Dermatosen. *Dermatol. Wochschr.* **137**: 257.
2. WEBER, G. 1958. Über das Verhalten der Aldolase im Blutserum und der Hautblasenflüssigkeit bei Dermatosen. *Dermatol. Wochschr.* **137**: 737.
3. WEBER, G. & I. WILDNER. 1958. Über die Milchsäuredehydrogenase-Aktivität im Blut und der Hautblasenflüssigkeit bei Dermatosen. *Dermatol. Wochschr.* **138**: 767.
4. WEBER, G. & H. THEISEN. 1958. Zur Frage des Verhaltens der Glutaminsäure-Brenztraubensäure-Transaminase im Blut- und Hautblasenserum bei Ekzem und bullösen Dermatosen. *Arch. klin. Exptl. Dermatol.* **208**: 93.
5. WEBER, G. 1959. Glutaminsäure-Oxallessigsäure-Transaminase in pathologisch veränderter menschlicher Hornschicht. *klin. Wochschr.* **37**: 234.
6. WEBER, G. & H. THEISEN. 1959. Zum Nachweis enzymatischer Mechanismen in menschlicher Epidermis am Modell der subepidermalen Blase. *Arch. klin. Exptl. Dermatol.* **208**: 459.
7. WEBER, G. 1959. Über enzymatische Mechanismen in menschlicher Epidermis. *Münch. med. Wochschr.* **101**: 1872.
8. WEBER, G. 1960. Vergleichende fermentchemische Untersuchungen im Blut, Hautblasenserum, Epidermishomogenat und in Psoriasissschuppen. *Arch. klin. Exptl. Dermatol.* **211**: 183.
9. WEBER, G. 1961. Vergleichende bioenzymatische Untersuchungen in der Hornschicht von Mensch und Schuppentieren (Squamaten). *Naturwiss.* In press.
10. WEBER, G. 1961. Der Einfluß von Triamcinolon auf Fermentmechanismen bei parakeratotischer Verhornung. *Arch. klin. Exptl. Dermatol.* In press.
11. WEBER, G. 1960. The reaction of enzymes in the course of pathological keratinisation. *IVth Intern. Congr. Clin. Chem.* Edinburgh, Scotland.
12. WIELAND, TH., G. PFLEIDERER, I. HAUPT & W. WÖRNER. 1959. Über die Verschiedenheit der Milchsäuredehydrogenasen. *Biochem. Z.* **332**: 1.
13. PFLEIDERER, G. & E. D. WACHSMUTH. 1961. Die Heterogenität der Lactatdehydrogenase in Entwicklungsgeschichte und Pathologie des Menschen. *Klin. Wochschr.* In press.
14. HESS, B. 1960. Über das Protein der Lactatdehydrogenase im menschlichen Serum und Geweben. *Klin. Wochschr.* **38**: 1080.

MULTIPLE FORMS OF LACTIC DEHYDROGENASE IN TISSUES OF THE MOUSE: THEIR SPECIFICITY, CELLULAR LOCALIZATION, AND RESPONSE TO ALTERED PHYSIOLOGICAL CONDITIONS*

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There is increasing evidence that many enzymes exist in tissues and cells in multiple molecular forms (Markert and Møller, 1959). Of less certainty, however, is the question of differences between these multiple molecular forms in terms of their catalytic and physiological activities. Indeed Markert and Møller felt that these forms, termed isozymes, possessed identical substrate specificities although the pattern of the isozymes possessed by a tissue changed radically during development. The question of the degree of heterogeneity existing between the multiple molecular forms of an enzyme is one of considerable importance. Such heterogeneity may be reflected in several diverse ways: (1) differences in substrate specificity, (2) differences in capacity to utilize cofactor analogs, (3) differences in sensitivity to inhibitors, (4) differences in response to varied physiological conditions, or (5) differences in the site of localization within or between cells. An examination of these possible points of difference between the multiple forms of lactic dehydrogenase in the tissues of the mouse comprises the content of this paper.

Materials and Methods

All work was carried out using tissues from BALB/c Jax mice. Tissues for electrophoretic study were homogenized in 2 parts (w/v) 0.9 per cent sodium chloride, using all-glass homogenizers. These homogenates were centrifuged at 4° C. for 10 min. at 1000 g to remove cell debris. The supernatant material from this centrifugation was employed for electrophoresis. Electrophoresis was carried out in a starch matrix after the method of Smithies (1959), as modified by Markert and Hunter (1959). The starch matrix† was prepared using 0.03 M boric acid-sodium hydroxide buffer at pH 8.5. The bridge buffer was similar in composition but was 10 times as concentrated. Electrophoresis was carried out for 6 hours at room temperature (ca. 25° C.), using a voltage drop of 6 v/cm. along the length of the starch slabs. For cytochemical study, 30 λ of the above supernatant material was applied to a 24 \times 6 mm. strip of Whatman No. 1 filter paper, and the strip was inserted into a transverse slit in the starch slab. For quantitative study of eluted enzyme, 60 λ of the supernatant was applied to a double-paper insert of the same size.

Cytochemical visualization of sites of lactic dehydrogenase activity in starch slabs was achieved by a modification of the method proposed by Dewey and Conklin (1961). The substrate mixture contained the following final concen-

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† Obtained from Connaught Medical Research Laboratories, Toronto, Ont., Canada.

trations of reactants: 0.025 *M* tris(hydroxymethyl)aminomethane* buffer pH 7.4, 0.1 *M* (*l*) lactic acid† [or one of the following, (*d*) lactic acid,† (*dl*) alpha-hydroxybutyric acid,* (*dl*) alpha-hydroxycaproic acid,* (*dl*) alpha-hydroxyisobutyric acid,* (*dl*) alpha-hydroxyisovaleric acid,* or (*dl*) beta-phenyllactic acid*]; 0.001 *M* diphosphopyridine nucleotide (DPN),* or deamino-diphosphopyridine nucleotide (DeamDPN);‡ 0.5 mg. per ml. nitro-blue tetrazolium (NBT);§ 0.005 *M* potassium cyanide; and 20 μ gm. per ml. phenazine methosulfate (PMS).* Starch slabs for incubation in this substrate mixture were sliced into 2 equal thicknesses with the bottom half always used for analysis. This bottom half was again divided longitudinally into equal halves so that 2 identical strips were available for comparative purposes. These strips were incubated at room temperature (ca. 25° C.) in total darkness for 30 min. The results were recorded photographically, using constant conditions of exposure and development.

In cases where quantitative estimation of enzyme activity was to be made a 1-mm. strip was sliced from the edge of the starch slab and incubated in the above substrate mixture [containing (*l*) lactic acid] for 30 min. or less. This developed strip then served as a guide to the position of the corresponding activity sites in the remaining portion of the starch slab. These sites were cut out, using a jig that produced a 3-mm. slice to yield a starch block 3 mm. \times 6 mm. \times 23 mm. The 3-mm. slice was barely thicker than the thinnest bands observed in the tissues chosen for quantitative analysis (see below). These starch blocks were frozen at -10° C. and held for analysis. For analysis a block was placed in 1.0 ml. 0.9 per cent sodium chloride and, after thawing, was homogenized in an all-glass homogenizer to break up the gel structure. The resultant slurry was centrifuged at 4° C. at 1500 g for 15 min., and the supernatant material reserved for assay. The substrate mixture for quantitative analysis contained the following final concentrations of reactants (in volume of 3 ml.): 0.033 *M* tris(hydroxymethyl)aminomethane buffer pH 7.4; 0.003 *M* potassium cyanide; 0.016 *M* substrate material; and 0.00066 *M* DPN or DeamDPN. One-hundred λ of the above supernatant material was added to a cuvette containing all reactants except DPN or DeamDPN. After 30 sec. the reaction was started by the addition of the appropriate nucleotide. Change in optical density due to the formation of reduced nucleotide was measured at 340 m μ over a 1-min. period at 25° C., using a Beckman Model DU spectrophotometer. Optical density change was linear over this period of measurement. No-enzyme and no-substrate controls were provided. Quantitative determinations were made in duplicate on material derived from a minimum of 2 electrophoretic separations. All quantitative results are reported as mean values.

Methods of cytochemical study of lactic dehydrogenase distribution in tissue sections were identical to those employed by Allen and Slater (1961) except that 0.025 *M* tris(hydroxymethyl)aminomethane buffer was substituted for phosphate buffer.

* Obtained from Sigma Chemical Co., St. Louis, Mo.

† Obtained from the California Corporation for Biochemical Research, Los Angeles, California.

‡ Obtained from Pabst Brewing Co., Newark, N.J.

§ Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Results

Tissue specific patterns of multiple forms of lactic dehydrogenase. Examination of the major organs of the mouse indicated a high degree of heterogeneity in terms of the array of multiple forms of lactic dehydrogenase possessed by a particular structure. On the basis of this comparative study it was possible to distinguish 9 electrophoretically distinct sites of lactic dehydrogenase activity. No single tissue or organ possessed all 9 sites, and none failed to show at least 1 site. On the basis of this comparative study it was possible to choose 4 tissues that showed 1 or more of the 9 sites in moderate to high activity. This representative set included skeletal muscle, large intestine, kidney, and testis. The distribution of sites of lactic dehydrogenase activity in these representative tissues is shown in FIGURES 1 and 2. Sites A2 and A4, derived from large intestine, were close in their electrophoretic mobility to sites A1 (skeletal muscle) and A3 (skeletal muscle). However, study of starch slabs containing half-paper inserts and comparing kidney with large intestine or skeletal muscle with large intestine clearly indicated a difference in the mobilities of sites A1 and A2, and A3 and A4. Site C1 was very slow migrating and remained near the origin. The separation of the remaining sites proved no problem. It is probable that the 9 electrophoretically distinct sites separated represent the minimum number since, in certain cases, the presence of diffuse bands precluded positive identification of site position. Thus placenta, mammary gland, coagulating gland, seminal vesicle, parotid gland, and liver possessed diffuse sites in the areas occupied by sites A1 and A2 or A3 and A4 that were impossible to assign to one position or another.

The substrate specificity of the nine sites of lactic dehydrogenase activity. Study of the substrate specificities of the sites of lactic dehydrogenase depended in part upon the ability of the cytochemical reaction system to reflect activity levels accurately. Visual grading of site activity was based upon a scale in which grade 1 represented highest activity and grade 5 represented barely detectable activity. The results of such visual grading when (*l*) lactic acid was the substrate material may be seen in FIGURE 1, to which the intensity of the sites shown photographically in FIGURE 2 may be compared. Thus, sites A1, A6, and A7 showed comparable activity of grade 1; site C1 showed slightly lower activity of grade 2; sites A2, A4, A5 showed activity of grade 3; and sites C2 and A3 showed activity of grade 4. Quantitative estimations of eluted enzyme (TABLE 1) closely paralleled estimations of band intensity by visual inspection.

When the activities of the various sites against (*dl*) alpha-hydroxybutyric acid were compared to the activities against (*l*) lactic acid, distinct differences in substrate specificity among the sites became apparent (FIGURE 1). Activity of all sites with the exception of C2 was either depressed or abolished by the substitution of (*dl*) alpha-hydroxybutyric acid. Site C2 was as active against this latter material as against (*l*) lactic acid. Sites A2 and A4 showed no detectable activity against (*dl*) alpha-hydroxybutyric acid. The remaining sites showed easily discernible but diminished activity against (*dl*) alpha-hydroxybutyric acid. Quantitative determinations confirmed the trend of the cytochemical observations (TABLE 1).

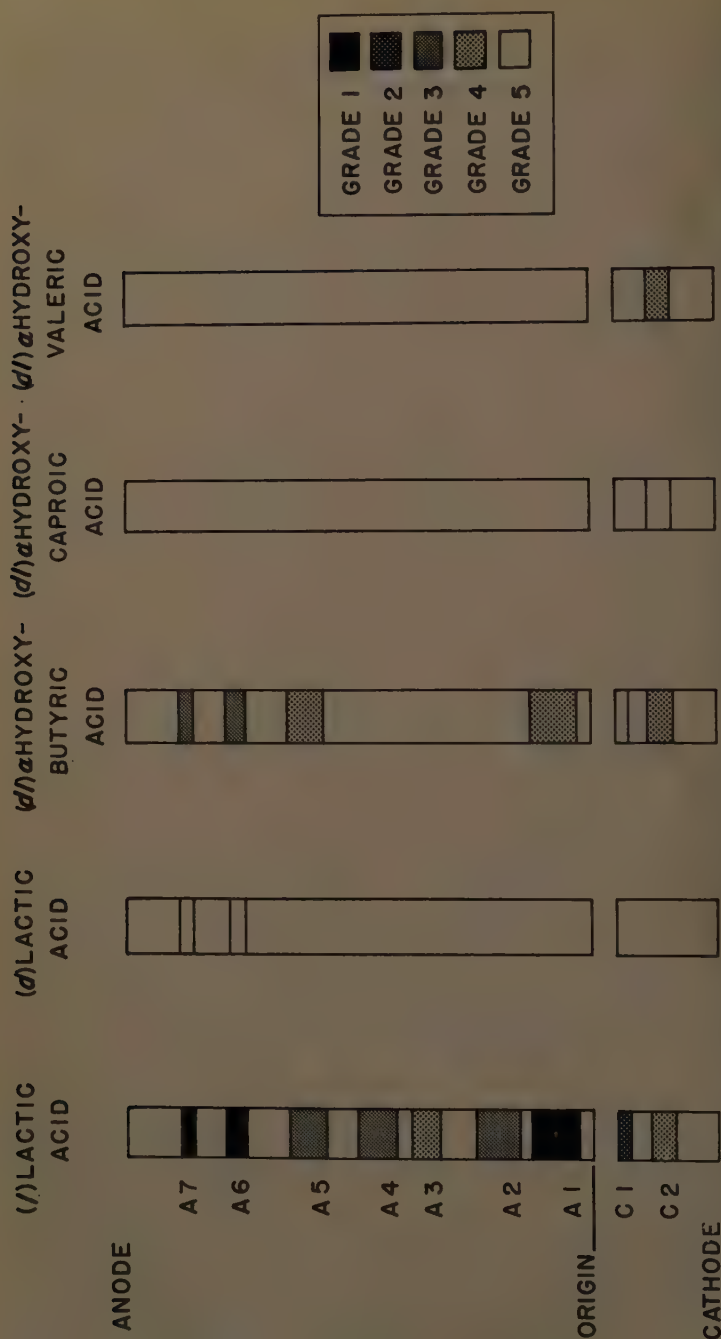
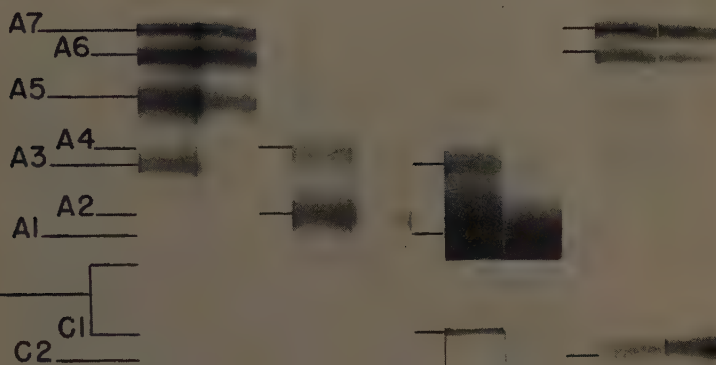


FIGURE 1. The graph is a diagrammatic representation of the activities of the various sites of lactic dehydrogenase activity based upon visual grading. Grade 1 represents highest activity, and grade 5 represents minimal activity. Sites A5, A6, and A7 were derived from kidney; sites A1, A3, and C1 were derived from skeletal muscle; sites A2 and A4 were derived from large intestine; and site C2 was derived from testis. The diagram expanded approximately two times along the vertical axis.

ANODE



CATHODE

a' b c' d e' f g' h

FIGURE 2. The activities of the sites of lactic dehydrogenase against (*l*) lactic acid in the presence of DPN (left member of each pair) and DeamDPN (right member of each pair). Strips *a* and *b* derived from kidney; strips *c* and *d* derived from large intestine; strips *e* and *f* derived from skeletal muscle; and strips *g* and *h* derived from testis. Enlarged 1:1 from 35 mm. negative material. All conditions of exposure and processing were held to constant values.

TABLE 1
ACTIVITY OF ELUTED ENZYME AGAINST VARIOUS SUBSTRATES

Site	(<i>l</i>) Lactic acid*	(<i>d</i>) Lactic acid† (%)	(<i>dl</i>) α Hydroxy- butyric acid† (%)	(<i>dl</i>) α Hydroxy- caproic acid† (%)	(<i>dl</i>) α Hydroxy- valeric acid† (%)
A7	0.017	0	20.1	0	0
A6	0.018	0	14.8	0	0
A5	0.009	0	9.1	0	0
A4	0.007	0	0	0	0
A3	0.006	0	8.1	0	0
A2	0.008	0	0	0	0
A1	0.040	0	12.3	0	0
C1	0.012	0	26.2	0	0
C2	0.007	0	87.5	128.0	86.0

* Activity expressed as change in optical density per minute per 100 λ eluate.

† Activity against this substrate expressed as per cent of activity against (*l*) lactate control.

Substitution of (*dl*) alpha-hydroxycaproic acid or (*dl*) alpha-hydroxyvaleric acid for (*l*) lactic acid resulted in the loss of all anodal sites of activity, as well as that of site C1. Site C2, however, was as active against these substitutions as against (*l*) lactic acid. Quantitative determinations (TABLE 1) confirmed these cytochemical observations.

Substitution of (*d*) lactic acid for (*l*) lactic acid resulted in the loss of all activity except at sites A6 and A7 (FIGURE 1). These latter sites showed barely detectable activity (Grade 5). Quantitative determinations failed to detect activity against (*d*) lactic acid on the part of any site (TABLE 1).

Substitution of (*dl*) alpha-hydroxyisovaleric acid, (*dl*) alpha-hydroxyiso-

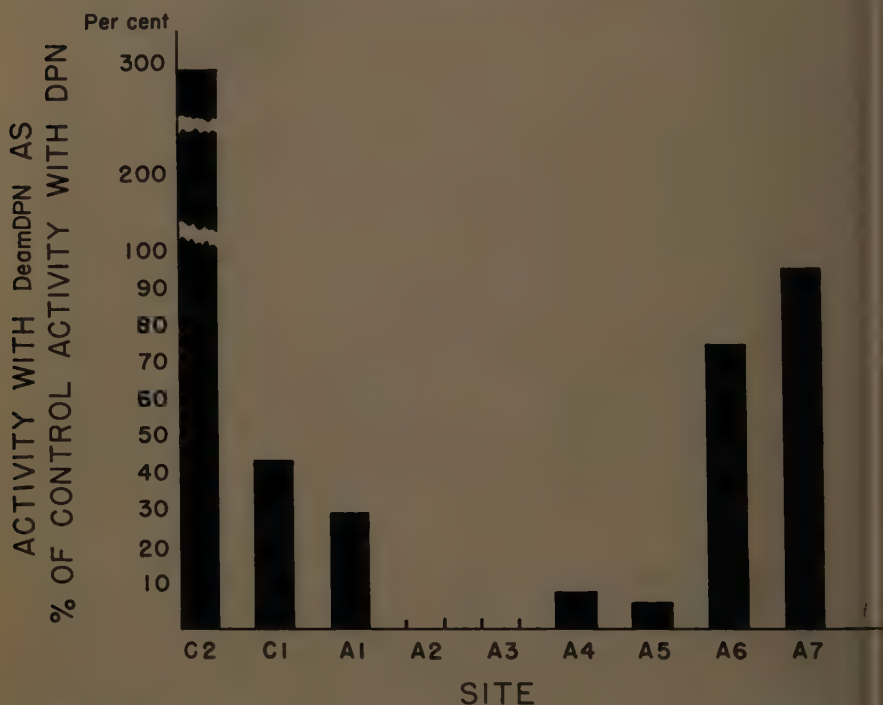


FIGURE 3. The activities of the various sites against (*l*) lactic acid in the presence of DeamDPN expressed as per cent of activity of the site against (*l*) lactic acid in the presence of DPN. The derivation of sites is as in FIGURE 1.

butyric acid, or (*dl*) beta-phenyllactic acid for (*l*) lactic acid resulted in the loss of all anodal sites, as well as site C1. Cathodal site C2 attacked (*dl*) alpha-hydroxyisovaleric acid at a rate slightly less than that against (*l*) lactic acid. Activity against (*dl*) beta-phenyllactic acid was of low order (grade 5), and (*dl*) alpha-hydroxyisobutyric acid yielded no perceptible reaction. Quantitative determinations were not made with this latter group of materials.

Effect of deamino-diphosphopyridine nucleotide upon the activity of the lactic dehydrogenase sites. Cytochemical comparison of the activities of the various sites against (*l*) lactic acid in the presence of DPN or DeamDPN indicated differences to the extent that the analogue could substitute for the natural

cofactor. Sites A7, A6, and A1 showed equal cytochemical activity (FIGURE 2) with both nucleotides. Sites A5 and C1 showed moderate depression of activity with DeamDPN. Sites A4, A3, and A2 were drastically inhibited by substitution of the analogue. Site C2, in contrast, was markedly enhanced in activity when DeamDPN was used. Quantitative determinations confirmed and extended these cytochemical observations (FIGURE 3). In this case the activities of sites A7 and A6 were seen to be only slightly affected by the substitution of DeamDPN, but the activities of sites A2, A3, A4, and A5 were

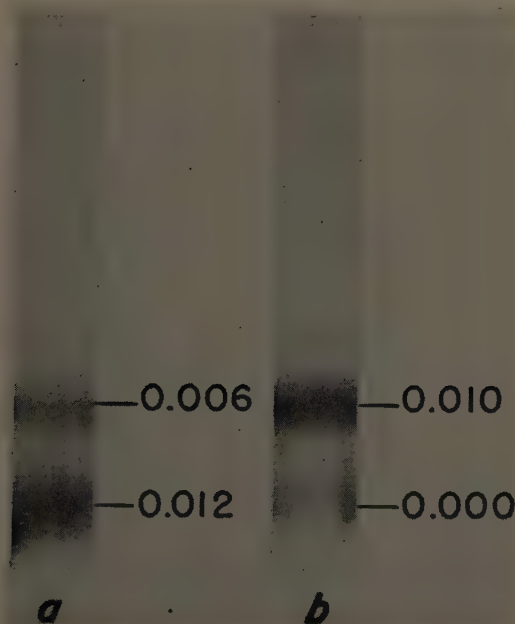


FIGURE 4. The comparison of activities against (l) lactic acid of sites derived from the mammary gland of an ovariectomized animal (a) with activities of sites derived from the mammary gland of a lactating animal (b). The figures to the right of each column refer to the change in optical density per minute produced by 100 λ of eluted enzyme in quantitative determinations. Enlarged 1:1 from 35 mm. negative material. The conditions of exposure and development are identical to those employed in FIGURE 2.

drastically depressed. Sites C1 and A1 showed moderate inhibition of activity with DeamDPN. The activity of site C2 was increased by nearly threefold when the analogue was substituted for DPN.

The effect of inhibitors upon the activities of the lactic dehydrogenase sites. Inclusion of 0.001 *M* pyruvic acid or oxamic acid in cytochemical substrates containing DPN and (l) lactic acid resulted in general depression of the activities of the various sites. No differential effect of these materials could be discerned by visual inspection.

The heterogeneity of the response of the various sites to altered physiological conditions. Examination of certain tissues under altered physiological conditions suggested that the response of the various sites was heterogeneous. Comparison of lactating mammary gland with mammary gland from ovari-

ectomized animals (FIGURE 4) indicated that the sites in this tissue responded differently to these conditions. Both cytochemical and quantitative determinations indicated that the slowest migrating site had highest activity in tissue from ovariectomized animals, whereas the faster migrating site showed highest activity in tissue from lactating animals. Thus there was a complete reversal of the quantitative relations between these sites under the differing physiological conditions. The diffuse nature of the sites in mammary gland precluded definite location as to position. Attempts to visualize shifts in activity within or between cells in sections of mammary gland under these conditions met with failure.

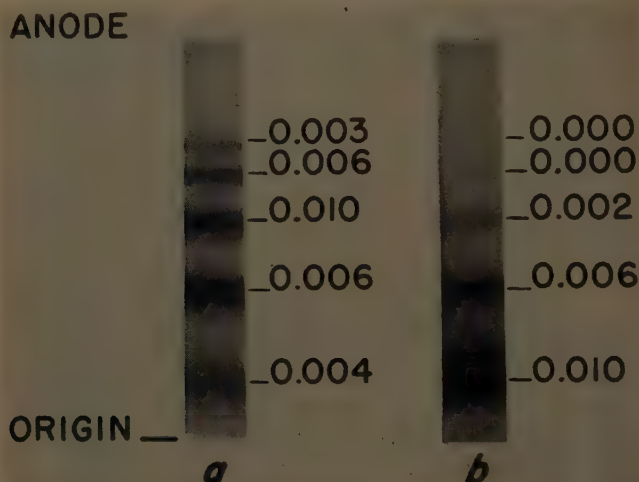


FIGURE 5. The comparison of activities against (*l*) lactic acid of sites derived from uteri from ovariectomized animals (*a*) with activities of sites derived from uteri from ovariectomized animals treated with 1 μ gm. estradiol benzoate daily for 5 days (*b*). The figures to the right of each column refer to the change in optical density per minute produced by 100 λ of eluted enzyme in quantitative determinations. The sites represented are A1, A3, A5, A6, and A7. Land Polaroid direct positive 1:1, band intensities are not strictly comparable to those in FIGURES 2 and 4.

In another instance, when the uterus from ovariectomized animals was compared to the uterus from ovariectomized animals that had been injected with 1 μ gm. estradiol benzoate daily for a week, a high degree of heterogeneity in the response of various sites of lactic dehydrogenase activity was noted (FIGURE 5). Site A1 in tissues from estrogen-replaced animals was markedly increased in activity, site A2 showed activity equal to that seen in ovariectomized animals, and the activities of sites A3, A4, and A5 were greatly depressed when compared to ovariectomized controls. Attempts to follow similar heterogeneity of response on the part of component cells in the uterus under these treatment conditions, using microscopic cytochemical methods, revealed no clear-cut changes in activity that could be related to the changes noted in electrophoretic studies.

In the case of epididymis, comparison of material derived from normal males

and from males subject to section of the vasa efferentia indicated that this treatment resulted in the total loss of site C2 (FIGURE 6), but had no effect upon the activity of any other site. Identical results were obtained when epididymides from castrated animals were compared to those from normal animals.

The possibility that certain sites may be localized to specific regions of cells. In

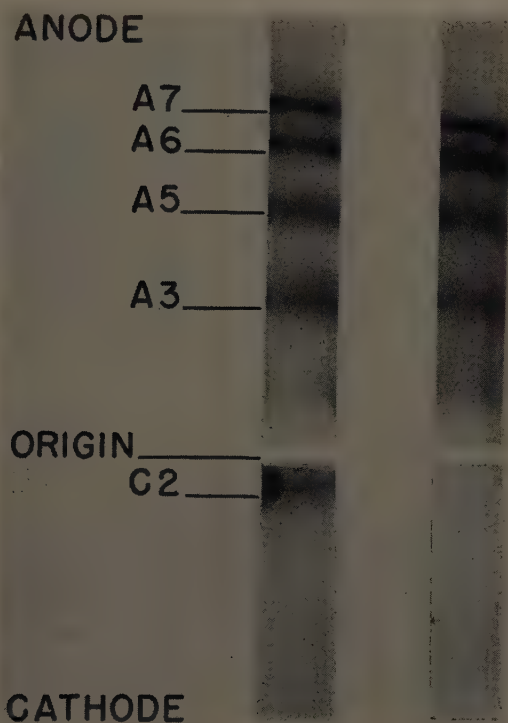


FIGURE 6. The comparison of activities against (L) lactic acid of sites derived from epididymides from normal animals (left) and from animals vasectomized 30 days (right). Enlarged 1:1 from 35 mm. negative material. The photographic representation of site intensities is not comparable to that in FIGURES 2 and 3, due to the necessity of processing alterations to show sites with weak activity.

In the case of epididymis it appeared that a site visualized in electrophoretic studies could be assigned to a particular cellular locale. Previous work (Allen and Slater, 1961) demonstrated a very distinct localization of lactic dehydrogenase activity to the apical regions of epithelial cells lining the body segment of the epididymal canal (FIGURE 7a). In other portions of the organ, activity was of moderate levels and was distributed homogeneously in the cytoplasm of epithelial cells. Following section of the vasa efferentia (henceforth referred to as vasectomy) for a period of 14 days, the apical activity in the body cells was lost, but levels of activity in the remaining cytoplasm were not affected (FIGURE 7c). Quantitative studies confirmed these cytochemical observations. The loss of epididymal site C2, previously noted, following vasectomy suggested

that a relationship might exist between this site and the apically distributed lactic-dehydrogenase activity observed in sections of epididymis.

Support for this contention was derived from studies of the substrate specificity of the apical lactic dehydrogenase in sections of epididymis in comparison with the substrate specificity of epididymal site C2. Substrate properties identical to those observed for testis site C2 were noted for epididymal site C22.

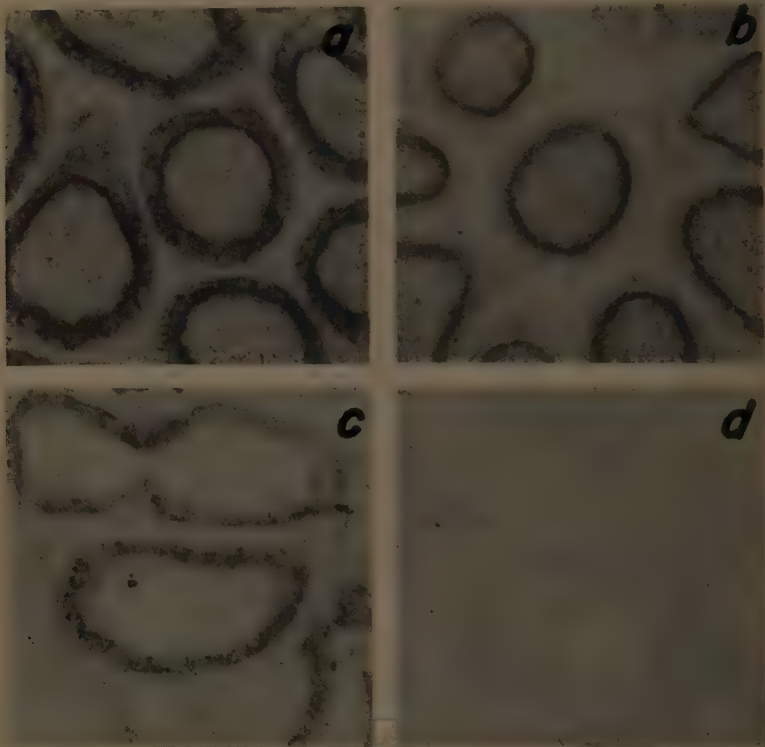


FIGURE 7. (a) The distribution of activity against (l) lactic acid in the epithelial cells of the body segment of epididymis from a normal animal. A marked apical distribution of activity is noted in these cells. The more basal cytoplasm contains moderate levels of homogeneously distributed activity. Nuclei, connective tissue cells, and sperm cells are unreactive. Five min. incubation at 37° C. with nitro-blue tetrazolium, 10 μ cryostat section. Land Polaroid direct positive, Wratten G filter, $\times 165$.

(b) The distribution of activity against (dl) alpha-hydroxycaproic acid in the epithelial cells of the body segment of epididymis from a normal animal. A very distinct apical distribution of activity is seen. The more basal portions of the cytoplasm are unreactive in contrast to the moderate activity seen in the same region in a. Nuclei, connective tissue cells, and sperm cells are unreactive. The preparation and photography are identical to that used in a.

(c) The distribution of activity against (l) lactic acid in the body cells of epididymis from an animal vasectomized for 30 days. The apical distribution of enzyme is no longer visible (compare to a) but moderate levels of activity are homogeneously distributed in the general cytoplasm. Nuclei and connective tissue cells are unreactive. The preparation and photography are identical to that used in a.

(d) The distribution of activity against (dl) alpha-hydroxycaproic acid in the body cells of epididymis from an animal vasectomized for 30 days. There is a complete loss of reaction in all tissue components. The preparation and photography are identical to that used in a.

except that activity levels were lower. The anodal sites of epididymis were inactive against any of the test substrates [except for (*l*) lactic acid] when incubation was for 30 min. Sites A6 and A7 showed barely perceptible activity with (*dl*) alpha-hydroxybutyric acid after 1 hour incubation. Low levels of activity in epididymal sites precluded elution and quantitative measurement of site activities. When similar tests of substrate specificity were made using sections of epididymis from normal animals, the apical regions of the epithelial cells lining the body segment of the canal showed equal intensity of reaction with (*l*) lactate, (*dl*) alpha-hydroxybutyric acid, (*dl*) alpha-hydroxyvaleric acid, and (*dl*) alpha-hydroxycaproic acid (FIGURE 7b). This cellular region did not react with (*d*) lactic acid. In all of these cases, with the exception of (*l*) lactic acid, no other area in these cells was reactive (FIGURE 7b). Other cells, not restricted to the body region of the epididymis, and which gave moderate homogeneously distributed reactions with (*l*) lactic acid, were completely unreactive with the other test substrates.

Tissues from vasectomized animals were completely unreactive (FIGURE 7d) with the test substrates, with the exception of (*l*) lactic acid. This latter material yielded a moderate reaction in the general cytoplasm, which was comparable to that seen in these areas in tissues from normal control animals (FIGURE 7c).

Experiments were carried out to determine if the loss of site C2 in vasectomized animals might be due to loss of sperm cells. When equal aliquots of whole homogenates, supernatant fractions (derived as usual), and washed sediments derived from the original centrifugation (washed 3 times in 0.9 per cent sodium chloride, centrifuged at $1000 \times g$ for 10 min. after each wash, and resuspended in a volume of 0.9 per cent sodium chloride equal to the original aliquot) were compared it was found that no activity was associated with the washed sediment. Thus it appears unlikely that the loss of site C2 in electrophoretic studies of epididymides from vasectomized animals is due simply to absence of sperm cells.

Discussion

The data accumulated in this study suggest that a considerable degree of heterogeneity exists between the various molecular forms of lactic dehydrogenase in the tissues of the mouse. In terms of substrate specificity it appears that these molecular forms do not have identical activities against the test substrates employed. The most conservative interpretation, in which minor differences in activity are ignored, indicates that the sites of lactic dehydrogenase discriminated may be separated into 3 groups or sets. Site C2 is clearly to be differentiated from the remaining sites on the basis of its capacity to dehydrogenate all of the test substrates, with the exception of (*d*) lactic acid. Similarly sites C2 and C4 may be differentiated from their congeners on the basis of their failure to oxidize any test substrate with the exception of (*l*) lactic acid. The remaining sites form a group of enzymes that show appreciable activity only against (*l*) lactic acid and (*dl*) alpha-hydroxybutyric acid. Quantitative determinations indicated, within the group, variation in the relative rates with which (*dl*) alpha-hydroxybutyric acid was dehydrogenated. The validity of these minor variations must await further determinations and

statistical analysis. A suggestion of cleavage within this latter group rests in the capacity of sites A6 and A7 to oxidize (*d*) lactic acid. All other sites failed to react with this compound. These results are in contradistinction to those of Markert and Møller (1959). These investigators felt that activity of the multiple forms of lactic dehydrogenase was identical against members of a similar battery of test substrates. The differences obtained in the present study may be due to the use of an improved substrate mixture for visualization of activity on starch. The employment of phenazine methosulfate and nitroblue tetrazolium in the Dewey and Conklin (1961) substrate would be expected to yield more critical rendering of activity values, due to the use of a more sensitive electron acceptor (NBT) and an artificial electron carrier (PMS), rather than natural diaphorase. Quantitative studies confirmed the accuracy with which the cytochemical system reflected activity levels of the various sites. At the present time no other comparable studies are known to me that deal with the substrate specificity of multiple forms of lactic dehydrogenase derived from vertebrate tissues. However, Singer *et al.* (1960) suggest that there may be multiple forms of lactic dehydrogenase in yeast, which are distinguishable on the basis of their capacity to oxidize (*l*) lactic acid and (*d*) lactic acid when different electron acceptors are used. In the case of a hexosephosphatase derived from *E. coli*, Rogers and Reithel (1960) have been able to identify 3 molecular forms of the enzyme, each with different relative activities against various substrates. In other cases, multiple forms of enzymes separated by methods similar to those used in the present study appear to have identical substrate specificities. Press *et al.* (1960) found cathepsin D to occur in 10 electrophoretically distinct forms with identical substrate capacities. The multiple forms of esterases studied in *Tetrahymena* by S. L. Allen (1961) also appear to have identical substrate properties within an isozymic group. Certainly the problem of substrate specificity of multiple forms of enzymes is far from resolved. It is likely that a considerable degree of variation in the extent of substrate specificity between multiple forms of different enzymes exists, and no simple statement of these relations will suffice for their description.

The use of DeamDPN served to further differentiate the multiple forms of lactic dehydrogenase, and indicated that cleavage within the sets mentioned above is possible. The activities of sites A6 and A7 were only slightly affected by the substitution of the analogue, whereas activity in sites A2, A3, A4, and A5 was drastically reduced. Sites A1 and C1 were moderately inhibited in activity by the substitution, but the activity of site C2 was elevated. These results further contrast the properties of site C2, and also point to a difference in the properties of sites A6 and A7 and the properties of the remaining sites. The difference in properties of sites A6 and A7 was suggested by their capacity to oxidize (*d*) lactic acid (see above). The use of cofactor analogues in the study of dehydrogenases separated electrophoretically has not been reported previously. However Kaplan *et al.* (1960) used analogues of DPN to successfully distinguish lactic dehydrogenases derived from the same tissues of different species and different tissues of the same species. These results are interpretable on the basis that multiple forms of lactic dehydrogenases exist that have

different capacities for interaction with various analogues of nucleotide cofactors.

In the present study the use of inhibitors of lactic dehydrogenase failed to distinguish between the various multiple forms of lactic dehydrogenase. In other cases, inhibitor studies have served to differentiate between lactic dehydrogenases of different tissues (Pfleiderer *et al.*, 1958).

An important distinction between multiple forms of an enzyme may reside in differential responses to changes in physiological environment. The reciprocal behavior of the sites of lactic dehydrogenase activity seen when comparing mammary tissue from lactating and ovariectomized animals is illustrative of such a situation. Also the heterogeneity of site response demonstrated in comparisons of uterus from ovariectomized and estrogen-treated ovariectomized animals indicates a differential response of the components of a multiple enzyme system to physiological change. It is probable that these differential responses have important significance in terms of cellular function, and they may reflect the action of microenvironmental influences upon enzymes situated in different regions of the cell or, perhaps, in different cells. Similar heterogeneity of response between members of the family of nonspecific esterases resident in epididymis has been demonstrated by Allen and Hunter (1960). The changes in lactic dehydrogenase constituents of tissues during differentiation reported by Markert and Møller (1959) are doubtless due to analogous environmental alterations. Such differential responses suggest that multiple forms of enzymes, although they may have similar substrate specificities, may be instrumental in different physiological functions.

A final point related to the heterogeneity of multiple forms of lactic dehydrogenase (and other enzymes as well) is the possibility that various constituents of these groups are situated at different sites within the cell. Such topological heterogeneity might be expected to have important implications in terms of the activity of an enzyme within the cell. A differential situation could result in the exposure of an enzyme to different microenvironmental influences that would be expected to affect its function. Also specific localization of an enzyme could result in channeling biochemical reaction sequences. Indeed a great deal of present-day cytological research demonstrates the restriction of enzymatic capacities to this or that cell structure. The research of Greene (1961) demonstrates most clearly the specific situation of macromolecules and the importance of this for the completion of a biochemical reaction sequence (electron transport and oxidative phosphorylation). The cytochemical studies reported in the present paper indicate that one of the sites of activity seen in electrophoretic preparations (C2) may be restricted to a specific cellular region. Restriction of a particular member of an isozymic set to a specific cellular region has also been reported for esterase in *Tetrahymena* (S. L. Allen, 1961). Nace *et al.* (1961) have been able to localize serologically distinct lactic dehydrogenases to different regions of the frog oocyte. It is possible that many of the multiple forms of enzymes separable by electrophoresis may represent enzymes with specific cellular localizations.

The heterogeneity of distribution of multiple forms of lactic dehydrogenase seen between various tissues and organs raises the question that each site may

be resident in a given cell type and that the observed heterogeneity within tissues is due simply to a multiplicity of component cells. It is unlikely that this is true, as studies carried out on cultured fibroblasts (Tsao, 1960) showed a multiplicity of electrophoretically distinct lactic dehydrogenases. Also clonal lines of *Tetrahymena* (S. L. Allen, 1961) contain a number of esterase isozymes, and Nace *et al.* (1960) found four serologically distinct lactic dehydrogenases within frog ova.

On the basis of these studies it appears that a considerable degree of heterogeneity exists between different members of a multiple molecular series of lactic dehydrogenases. The differences between members of such a series are not as profound as those existing between hydrolases with low degree of substrate specificity (Markert and Hunter, 1959; Hunter and Burstone, 1960). In agreement with Markert and Møller (1959) it is felt that a continuum of properties does not exist between one enzyme and the next. However, it is felt that pronounced differences in properties do exist between members of the multiple series of lactic dehydrogenases from mouse tissues. These differences are extensive enough in certain instances (site C2 versus sites A2, and A4, and the remaining sites) to suggest that, within the lactic dehydrogenase series, sets or groups of enzymes exist with similar properties. It is perhaps proper to term these groups *isozymic sets* to indicate that the members possess similar characters. It is evident that the characterization of electrophoretically distinct macromolecules must rest upon several points of comparison rather than upon a single criterion such as substrate specificity.

Summary

The multiple forms of lactic dehydrogenase in tissues of the mouse have been investigated, using starch-gel electrophoresis combined with cytochemical and quantitative methods of analysis. A survey of the major organs of the mouse indicated that at least nine electrophoretically distinct lactic dehydrogenases were demonstrable. The properties of these sites were studied, using extracts of skeletal muscle, kidney, testis, and large intestine. The substrate specificities of the various sites, demonstrated in electrophoretic studies, differed in their capacity to oxidize (*l*) lactic acid, (*d*) lactic acid, (*dl*), alpha-hydroxybutyric acid, (*dl*) alpha-hydroxycaproic acid, and (*dl*) alpha-hydroxyvaleric acid. Thus one site had equal activity against all substrates with the exception of (*d*) lactic acid. Two sites oxidized only (*l*) lactic acid. The remaining sites had variable activity against (*dl*) alpha-hydroxybutyric acid, two of these oxidized (*d*) lactic acid, and none was active against (*dl*) alpha-hydroxycaproic acid, or (*dl*) alpha-hydroxyvaleric acid. The use of deamino-diphosphopyridine nucleotide served to contrast further these multiple forms of lactic dehydrogenase. Certain sites were affected only slightly by the substitution of the analogue, others were drastically depressed in activity, and one site was increased in activity.

The behavior of the various sites to physiological modification was examined. The behavior of the various sites to such modification was heterogeneous. In one case, by taking advantage of physiological changes and substrate specificity differences, it was possible to localize a site observed in electrophoretic prepara-

tions to a particular locale within the epithelial cells lining the body segment of the epididymal canal.

These results are discussed in terms of the physiological significance of multiple forms of enzymes for tissue and cell function.

References

- ALLEN, J. & R. HUNTER. 1959. A histochemical study of enzymes in the epididymis of normal, castrated and hormone replaced castrated mice separated by zone electrophoresis in starch gels. *J. Histochem. Cytochem.* **8**: 50-57.
- ALLEN, J. & J. SLATER. 1961. A cytochemical analysis of the lactic dehydrogenase-diphosphopyridine nucleotide-diaphorase system in the epididymis of the mouse. *J. Histochem. Cytochem.* **9**: 221-233.
- ALLEN, S. L. 1961. Genetic control of the esterases in the protozoan *Tetrahymena pyriformis*. *Ann. N.Y. Acad. Sci.* **94**(3): 753-773.
- DEWEY, M. & J. CONKLIN. 1961. Starch gel electrophoresis of lactic dehydrogenases from rat kidney. *Proc. Soc. Exptl. Biol. Med.* **105**: 492-494.
- GREEN, D. & Y. HATEFI. 1961. The mitochondrion and biochemical machines. *Science*. **133**: 13-19.
- HUNTER, R. & M. BURSTONE. 1960. The zymogram as a tool for the characterization of enzyme substrate specificity. *J. Histochem. Cytochem.* **8**: 58-62.
- KAPLAN, N., M. CIOTTI, M. HAMOLSHY & R. BIEBER. 1960. Molecular heterogeneity and evolution of enzymes. *Science*. **131**: 392-397.
- MARKERT, C. & F. MØLLER. 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. *Proc. Natl. Acad. Sci.* **45**: 753-763.
- MARKERT, C. & R. HUNTER. 1959. The distribution of esterases in mouse tissues. *J. Histochem. Cytochem.* **7**: 42-49.
- NACE, G., T. SUYAMA & N. SMITH. Early development of special proteins. Symposium of Internat. Inst. of Embryol. Pallanza, Italy. In press.
- PFLEIDERER, G., D. JECKEL & T. WIELAND. 1958. Über den Wirkungsmechanismus der Milchsäure-dehydrogenasen. II. Zur Frage des Zinkgehalts und der SH-Gruppen. *Biochem. Z.* **330**: 296-302.
- PRESS, E., R. PORTER & J. CEBRA. 1960. The isolation and properties of a proteolytic enzyme, cathepsin D, from bovine spleen. *Biochem. J.* **74**: 501-514.
- ROGERS, D. & F. REITHEL. 1960. Acid phosphatases of *Escherichia coli*. *Arch. Biochem. Biophys.* **89**: 97-104.
- SINGER, T. P., E. KEARNEY, D. GREGOLIN, E. BOERI, M. RIPPA & E. FORD. 1960. On the multiplicity of lactic dehydrogenase in yeast. *Biochem. Biophys. Research Com.* **3**: 428-434.
- SMITHIES, O. 1959. Zone electrophoresis in starch gels and its application to studies of serum proteins. In *Advances in Protein Chemistry*. **14**: 65-113. C. Anfinsen, M. Anson, K. Bailey, and J. Edsall, Eds. Academic Press. New York, N.Y.
- TSAO, M. 1960. Heterogeneity of tissue dehydrogenases. *Arch. Biochem. Biophys.* **90**: 234-238.

Discussion of the Paper

A. SAMUELS (*Department of Pathology, Dartmouth Medical School, Hanover, N.H.*): Was it possible to make any estimate of the time course of disappearance of any of the fractions during physiological manipulation, so that a comparison of this time and the turnover time of the protein could be made? This could possibly lead to insight as to whether the synthesis or the conformation of the protein was affected by the treatment.

FEEDBACK INHIBITION AND REPRESSION OF ASPARTOKINASE ACTIVITY IN *ESCHERICHIA COLI**

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In recent years significant advances have been made in our knowledge concerning cellular regulation of biosynthetic metabolism. It has been established, for example, that specific, automatic controls of pathways leading to the synthesis of various amino acids, purines, and pyrimidines are exerted by excessive accumulation of end metabolites. At least two different mechanisms of control have been recognized. In the first, referred to as "repression," a high concentration of the end metabolite causes a repression of the synthesis of various enzymes involved in its formation.^{1,2} In the second mechanism, referred to as "feedback" control, a high concentration of the ultimate end product causes a specific inhibition of the enzyme catalyzing usually the first step in the metabolic sequence leading to its formation.³ It is a consequence of either type of regulation that excessive accumulation of a particular end product will automatically result in decreased activity of the metabolic pathway involved in its biosynthesis. The generality of these regulatory mechanisms having been established, certain situations are presented that provoke speculation. One such situation exists in the intermediary metabolism of aspartic acid summarized in FIGURE 1. Here it can be seen that aspartate, aspartyl-P, and aspartic semialdehyde are common intermediates in the biosynthesis of three different amino acids, lysine,⁴ threonine,^{4,5,6,7} and methionine.^{4,8}

It is therefore obvious that a difficulty may arise if this metabolic pathway is regulated by repression or feedback inhibition of the enzyme that catalyzes the first step in the metabolic sequence. With such control, an excessive accumulation of any one of the three ultimate end product amino acids could cause a decrease in the production of aspartyl-phosphate, which might result in a deficiency in the production of the other two amino acids.

In an effort to investigate this unique situation in greater detail an analysis of the aspartokinase system in *E. coli*, HfrC, has been undertaken. The aspartokinase activity of sonic extracts was determined by the method of Black,² which involves measurement of the asparthydroxamate that accumulates when aspartate and adenosine-triphosphate (ATP) are incubated with the enzyme in the presence of hydroxylamine. Under these incubation conditions, aspartokinase catalyzes the phosphorylation of aspartate, and the aspartyl phosphate so formed reacts nonenzymatically with hydroxylamine to form asparthydroxamate. The latter is easily quantitated by the method of Lipmann and Tuttle.¹⁰

In order to determine if the aspartokinase activity of crude sonic extracts is

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susceptible to feedback control, its inhibition by L-lysine, L-threonine, L-methionine, and DL-homoserine was investigated. Data from typical experiments are summarized in TABLE 1. The data show that aspartokinase is inhibited by all of these amino acids except methionine. Although the degree of inhibition varies somewhat with the growth conditions and extracting procedure, crude sonic extracts of *E. coli*, HfrC, are usually maximally inhibited 30 to 50 per cent by either lysine or threonine and 10 to 15 per cent by DL-homoserine. From other results not summarized in TABLE 1 it has been established that the inhibition exhibits high optical specificity; the D-optical

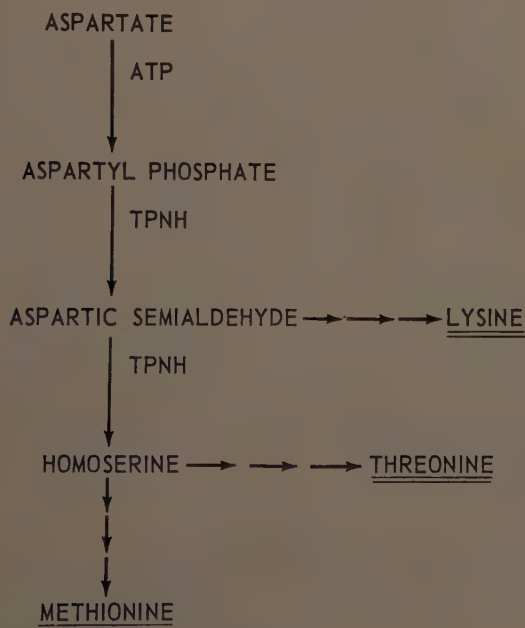


FIGURE 1.

isomers of lysine and threonine are inert as is also L-ornithine. Moreover, none of the other amino acids that are normal constituents of proteins is inhibitory.

At 10^{-2} *M* concentrations of lysine, threonine, and homoserine used in the experiments reported in TABLE 1, maximum inhibition for each amino acid is attained. The concentrations for one-half maximal inhibition are about 3 to 5×10^{-4} *M*. It is of special significance that when any two amino acids are added simultaneously, the total inhibition observed is roughly equal to the sum of that observed for each independently. This finding is consistent with the conclusion that the crude extracts contain three different aspartokinases, one of which is selectively inhibited by lysine, one by threonine, and one by homoserine. This conclusion is further supported by the data of TABLE 2 showing that fractionation of sonic extracts by ammonium sulfate precipitation results in a partial separation of the threonine and lysine sensitive activities.

Thus the protein fraction precipitating between 0 and 40 per cent ammonium sulfate saturation is greatly enriched with respect to the enzyme that is susceptible to inhibition by L-lysine, whereas the enzyme precipitating between 40 and 50 per cent ammonium sulfate saturation is almost specifically inhibited by

TABLE 1
THE INHIBITION OF ASPARTOKINASE BY VARIOUS AMINO ACIDS

Amino acid*	Inhibition of aspartokinase activity	
	Expt. 1 %	Expt. 2 %
Met	0.0	0.0
Lys	36.0	28.7
Thr	39.5	36.5
HS	7.3	12.2
Lys + Thr	85.0	76.4
Lys + HS	43.0	42.0
Thr + HS	46.0	43.0
Thr + HS + Lys	86.0	82.5
Lys + Met	36.5	—
Thr + Met	38.0	—
Lys + Met + Thr + HS	83.0	—

Each reaction mixture contained ATP, 10.4 mM; Tris HCl buffer (pH 8.1) 94 mM, MgSO_4 , 1.6 mM, β -mercaptoethanol 10 mM, L-aspartate, 10 mM, NH_2OH 800 mM, KCl, 800 mM, 0.9 mg. of crude sonic extract of *E. coli*, HfrC, and the other amino acids as indicated, 10 mM, except DL-homoserine which was 20 mM, total volume 1.0 ml. Incubation 26°C . 30 min. The reaction was stopped by the addition of 1.0 ml. of FeCl_3 reagent¹⁰ and, after incubation, the amount of asparthyroxamate present was measured at 540 $\text{m}\mu$ in a Beckman DU Spectrophotometer. Aspartokinase activity is defined as the optical density at 540 $\text{m}\mu$ $\times 1000$.

* The following abbreviations are used: L-methionine, Met; L-lysine, Lys; L-threonine, Thr; DL-homoserine, HS.

TABLE 2
SEPARATION OF THE LYSINE-SENSITIVE AND THE THREONINE-SENSITIVE ASPARTOKINASES

Enzyme preparation	Protein (mg.)	Total units	Lysine- inhibited units	Threonine- inhibited units	Homoserine- inhibited units
Sonic extract	1430	293,000	84,000	108,000	35,700
Streptomycin supernatant	956	293,000	86,000	108,000	35,000
0-40% (NH_4) ppt.	264	179,000	85,000	44,500	13,400
40-50% (NH_4) ppt.	234	80,000	2,700	64,500	27,000
0-40% (NH_4) ppt. (aged)	234	115,000	84,000	10,200	0

The aspartokinase activity was measured in the presence and absence of $10^{-2} M$ L-lysine, L-threonine, and $2 \times 10^{-2} M$ DL-homoserine. Otherwise the conditions were as in TABLE 1.

L-threonine. In the experiment described in TABLE 2, the homoserine-sensitive enzyme fractionated together with the L-threonine-sensitive enzyme; however, the nonidentity of these two enzymes is evident from other experiments in which the L-threonine enzyme has been obtained devoid of sensitivity toward homoserine. For the purpose of calculation, the amount of each enzyme activity reported in TABLE 2 was assumed to be equal to that fraction of the total aspar-

tokinase activity that is lost when the individual amino acid inhibitors are added at 10^{-2} *M* concentrations. The validity of this calculation is indicated by the fact that the total inhibition obtained when all three amino acids are present simultaneously is approximately the sum of that observed for each, individually (TABLE 1). It is further suggested by the excellent over-all recovery of the various activities during the fractionation with ammonium sulfate. It is not known if the failure to obtain 100 per cent inhibition when all three amino acids

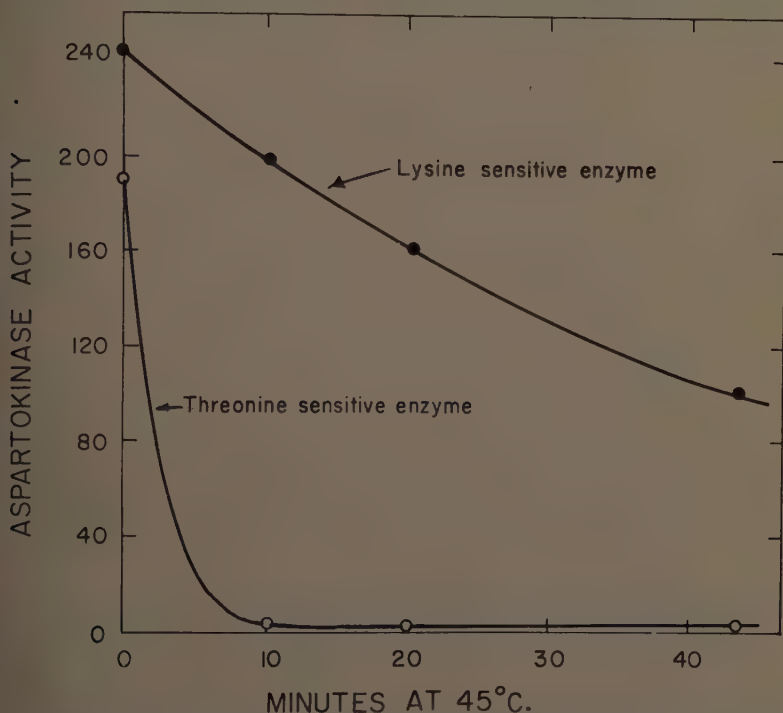


FIGURE 2. Heat stability of the lysine-sensitive aspartokinase and the threonine-sensitive aspartokinase. A streptomycin supernatant solution containing 15 mg. protein per ml. was heated at 45° C. for the indicated times. Then the aspartokinase activity of the heated enzyme (3 mg.) was determined as described in TABLE 1. The amounts of lysine-sensitive and threonine-sensitive enzyme are assumed to be equal to those fractions of the total aspartokinase activity that are lost when lysine (10^{-2} *M*) and threonine (10^{-2} *M*) respectively are present in the incubation mixtures.

are present is due to the existence of still another aspartokinase activity in crude extracts or whether one or more of the aspartokinases is not susceptible to complete inhibition under the experimental conditions.

Heat sensitivity. The lysine-sensitive and threonine-sensitive aspartokinases may be differentiated further on the basis of their stability to heat and to inactivation by autooxidation. When exposed to air at 0° C. in the absence of added sulfhydryl compounds, the threonine-sensitive enzyme activity is rapidly lost, whereas the lysine-sensitive enzyme is relatively more stable. The rate of inactivation is greatly accelerated by heat. Thus the data of FIGURE 2

show that heating at 45° C. for 10 min. causes almost complete inactivation of the L-threonine-sensitive enzyme, whereas the L-lysine-sensitive enzyme is only 10 to 20 per cent inactivated.

Inhibition analysis. The data reported in FIGURES 3*a* and 3*b* are from kinetic studies showing the extent of inhibition of the lysine-sensitive and threonine-sensitive aspartokinases as a function of substrate concentration. The $1/v$ versus $1/S$ plots in the presence and absence of these amino-acid inhibitors indicate that the inhibition by L-threonine is competitive whereas the inhibition by L-lysine is noncompetitive. From other experiments that will not be

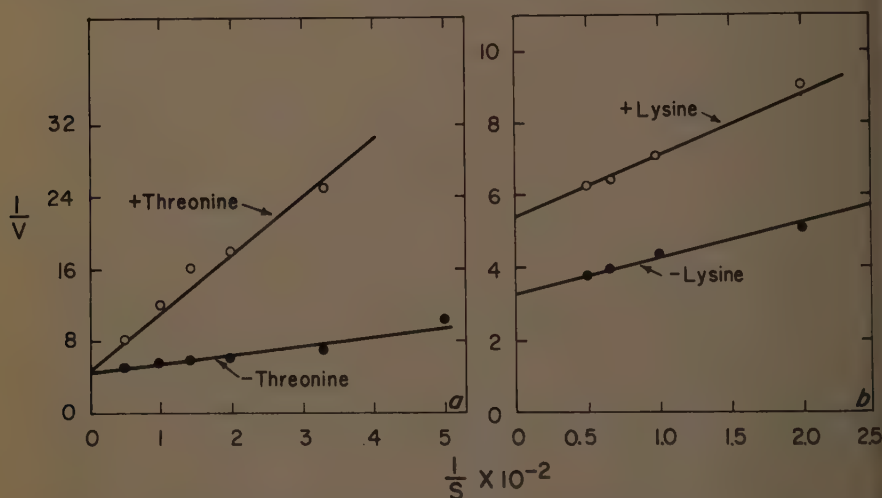


FIGURE 3. Inhibition analysis of the lysine- and threonine-sensitive aspartokinases. Key: v = amount of asparthydroxamate (expressed as optical density at 540 $m\mu$ of the ferric complex) formed in 30 minutes; S = moles of aspartate per liter. In experiment *a*, the assay mixtures contained 0.9 mg. protein from a 40 to 50 per cent ammonium sulfate fraction and, where indicated, 0.01 M L-threonine. In experiment *b*, 0.61 mg. of protein from a 0 to 35 per cent ammonium sulfate precipitate was the enzyme and, where indicated, 0.01 M L-lysine. Otherwise conditions were as described in TABLE 1.

described in detail it was demonstrated that the noncompetitive inhibition by L-lysine is reversible. The K_m 's for aspartate calculated from the data of FIGURE 3 are 3×10^{-3} for the lysine-sensitive enzyme and 2×10^{-3} for the threonine-sensitive enzyme.

Repression. The data presented thus far show that the aspartokinases of *E. coli* are subject to regulation through feedback inhibition by lysine, threonine, and homoserine. In order to determine if these enzymes are regulated also by repression, a study was made to determine if cellular concentrations of the various enzymes are influenced by the presence of high concentrations of threonine, lysine, methionine, and homoserine during growth. The results of a typical experiment summarizing the effects of threonine and lysine are presented in TABLE 3. It will be noted that the total aspartokinase activity of the sonic extract derived from bacteria grown on minimal medium is 184 units

per mg., and that this activity is inhibited 28.5 per cent by L-lysine and 46 per cent by L-threonine. When the organism is grown on a medium supplemented with 10^{-2} M L-threonine, the total aspartokinase activity is but slightly reduced and the susceptibility to inhibition by lysine and threonine is unchanged. On the other hand sonic extracts of *E. coli* grown on a minimal medium containing 10^{-2} M L-lysine contain a significantly lower aspartokinase level, and this aspartokinase is not at all susceptible to inhibition by L-lysine but is inhibited mainly by L-threonine. Finally the extract of organisms grown on a mixture of threonine and lysine contain even lower amounts of aspartokinase that is not inhibited by L-lysine. These data thus show that the synthesis of the aspartokinase which is selectively inhibited by L-lysine is completely repressed when the organism is grown in the presence of 10^{-2} M L-lysine and suggest that slight repression of the threonine-sensitive enzyme may occur when this amino acid is added to the growth medium, especially in the presence of L-lysine.

TABLE 3
REPRESSION OF ASPARTOKINASE BY GROWTH OF *ESCHERICHIA COLI* HfrC
ON L-THREONINE AND/OR L-LYSINE

Growth conditions	Aspartokinase (units/mg.)	Inhibition by	
		L-Lysine (%)	L-Threonine (%)
Minimal medium	184	28.5	46.0
Minimal medium + L-threonine, 10^{-2} M	164	30.0	43.0
Minimal medium + lysine, 10^{-2} M	137	0	74.0
Minimal medium + L-lysine, 10^{-2} M + L-threonine, 10^{-2} M	70	0	64.0

In other similar experiments it was established that growth on homoserine or methionine does not influence the composition of the aspartokinase activity of sonic extracts.

All of the data reported thus far have been derived from experiments on extracts of *E. coli*, HfrC. Other strains of *E. coli* have been found to possess the three different aspartokinases but the relative concentrations are variable. Thus in *E. coli*, strain W, the total aspartokinase level is somewhat higher than in the HfrC strain, and the relative amount of the threonine-sensitive enzyme is much lower (TABLE 4). With strain W, the lysine-sensitive and threonine-sensitive enzymes are both somewhat repressible when the organism is grown in the presence of these amino acids. Growth in the presence of high concentrations of both amino acids together produces an eight- to tenfold decrease in the total aspartokinase level. Curiously, the absolute amount of homoserine-sensitive activity is unchanged by growth on lysine and threonine.

In summary, extracts of *E. coli*, HfrC, have been found to contain two aspartokinases which are readily separated from each other by simple ammonium sulfate fractionation. One of these aspartokinases is specifically and non-competitively inhibited by L-lysine; its formation is completely repressed when

the bacterium is grown in the presence of L-lysine. The other aspartokinase is specifically and competitively inhibited by L-threonine; and its formation may be slightly repressed when the organism is grown in the presence of threonine and lysine.

Relatively smaller amounts of a third aspartokinase, which is specifically inhibited by DL-homoserine, are present also, but the properties of this enzyme have not been investigated in detail.

It should be emphasized that aside from differences in relative heat stability, the different aspartokinases are recognizable in crude extracts only on the basis of their differential inhibition and repression by L-lysine and L-threonine. They catalyze identical reactions involving the transfer of a phosphoryl group from ATP to the β -carboxyl group of aspartate to form β -aspartylphosphate. In studies not discussed, it was found that the separated enzymes cannot be differentiated on the basis of reaction kinetics or their responses to changes in aspartate, ATP, Mg^{++} or H^+ concentrations.

TABLE 4
REPRESSION OF ASPARTOKINASE BY GROWTH OF *ESCHERICHIA COLI*, STRAIN W,
ON L-THREONINE AND/OR L-LYSINE

Conditions of growth	Total (μ /mg.)	Aspartokinase activity inhibited by		
		Lysine (%)	Threonine (%)	Homoserine (%)
Minimal medium	305	72.5	8.0	1.5
Minimal medium + Thy*	308	75.3	1.7	4.5
Minimal medium + Lys + Thr	37	10.0	55.0	12.8
Minimal medium + Lys	105	19.0	61.0	—

* Abbreviations as in TABLE 1.

Discussion

In view of the fact that aspartyl phosphate formation represents the first step in various reaction sequences involved in the biosynthesis of several different end products (FIGURE 1), the presence of separate aspartokinases for each metabolic pathway is understandable from the standpoint of cellular regulation. In the absence of separate enzymes, the operation of "repression" and "feedback" mechanisms of control could lead to serious difficulties. Then an overproduction of one ultimate end product could result in a reduction of aspartokinase activity to a level below that necessary to provide adequate synthesis of other essential metabolites also derived from aspartyl phosphate.

Although the function of the homoserine-inhibited aspartokinase is unknown, it is a reasonable assumption that the lysine-inhibited and the threonine-inhibited enzymes of *E. coli* are synthesized in order to permit independent regulation of the pathways leading to the biosynthesis of lysine and of threonine respectively. A question that follows logically from this assumption is whether the biosynthesis of lysine and threonine involves two parallel biosynthetic pathways in which all of the common enzymatic steps are spatially separated

within the cell, or do the separate aspartokinases supply a common pool of aspartyl phosphate from which both amino acids are ultimately derived? If the latter situation is correct, then the function of separate enzymes, independently controlled by repression and feedback, would be to limit this control such that an overproduction of one amino acid could never cause a reduction in the aspartyl phosphate pool to a level below that necessary for the biosynthesis of the other amino acid. Repression or feedback control of the enzymes involved in later steps in their biosynthetic pathways might then direct the ultimate fate of the aspartyl phosphate pool. It is therefore of interest to determine the repression and inhibition of other enzymes involved in lysine and threonine biosynthesis.

In any event the results reported here suggest that the multiplicity of enzymes frequently observed to catalyze the same biochemical reaction in a single organism may represent situations in which the product of the catalyzed reaction is a common precursor in diverse metabolic pathways. In these instances it might prove fruitful to investigate the repression and feedback inhibition by reasonable end products.

References

1. COHN, M., G. N. COHEN & J. MONOD. 1953. *Compt. rend. acad. sci. Paris.* **236**: 746.
2. MONOD, J. & G. COHEN-BAZIRE. 1953. *Compt. rend. acad. sci. Paris.* **236**: 530.
3. UMBARGER, H. E. 1956. *Science.* **123**: 848.
4. ABELSON, P. H., E. T. BOLTEN & E. ALDOUS. 1952. *J. Biol. Chem.* **198**: 173.
5. COHEN, G. N. & M. L. HIRSCH. 1954. *J. Bacteriol.* **67**: 182.
6. NISMAN, B., G. N. COHEN, S. B. WIESENDANGER & M. L. HIRSCH. 1954. *Compt. rend. acad. sci. Paris.* **238**: 1746.
7. BLACK, S. & N. H. WRIGHT. 1955. *Amino Acid Metabolism.* : 591. Johns Hopkins Press. Baltimore, Md.
8. KALAN, E. B. & J. CEITHAML. 1954. *J. Bacteriol.* **68**: 293.
9. BLACK, S. & N. G. WRIGHT. 1955. *J. Biol. Chem.* **213**: 27.
10. LIPMANN, F. & L. C. TUTTLE. 1945. *J. Biol. Chem.* **159**: 21.

THE HETEROGENEITY OF THE COPPER-CONTAINING PROTEIN OF HUMAN PLASMA, CERULOPLASMIN

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Introduction

In the normal human adult ceruloplasmin, the blue copper-protein of plasma is present at a concentration of about 30 mg. per 100 ml. of plasma,¹ and accounts for almost all of the copper contained in plasma. There are many disease states accompanied by changes in the concentration of this protein, but the significance of these changes is virtually unknown. Deficiency of the protein occurs transiently in the neonatal state, in the nephrotic syndrome, and in sprue and kwashiorkor, principally,² but life-long deficiency, or even absence of ceruloplasmin is found, as an inherited characteristic, only in patients with Wilson's disease.³ In contrast to such *quantitative* variations, the work reported in this paper is concerned with *qualitative* variations of the protein.

Broman,⁴ Sankar,⁵ and Uriel⁶ first showed that ceruloplasmin may exist in more than one molecular form. Work in this laboratory,⁷ with purified ceruloplasmin prepared from the pooled plasma of 9,109 donors, indicated there were three chromatographically different ceruloplasmin fractions containing four electrophoretically variant forms. The chromatographic fractions were indistinguishable when compared with respect to copper content,¹ blue color, paraphenylenediamine (PPD)^{1,8} oxidase activity and the exchangeability of protein bound copper with ionic copper-64.⁹ Although the different electrophoretic mobilities of the chromatographic fractions of ceruloplasmin indicated possible differences in their amino acid compositions, such differences could not be detected by preliminary amino acid analyses.¹⁰

There are two principal results of our present studies. First, two fractions of ceruloplasmin are present in normal human adults. Second, two fractions of ceruloplasmin are present in newborn infants and in almost all patients with Wilson's disease despite the sharp reduction in concentration of ceruloplasmin found in these subjects.

Methods

Serum was prepared from venous whole blood collected from (1) 10 normal adult donors, and (2) 1 patient with Wilson's disease and from (3) the cords of 4 newborn infants. Ceruloplasmin was purified and concentrated as follows, and as outlined in TABLE 1.

The pooled serum from each source was diluted with twice its volume of 0.05 M phosphate buffer of pH 6.4 and adsorbed onto a diethylaminoethyl cellulose (DEAE)¹¹ column* previously equilibrated with a solution of 0.05 M sodium chloride in 0.05 M phosphate buffer of pH 6.4. The column was then thoroughly washed with the latter solution. The blue ceruloplasmin

* Cellex-D Anion Exchange Cellulose, Bio-Rad Laboratories, Richmond, Calif.

TABLE I

PURIFICATION OF HUMAN CERULOPLASMINS FROM SERUM

Chromatography	Material	OD $\frac{1\text{ cm.}}{610\text{ m}\mu}$	Purity of ceruloplasmin %
		OD $\frac{1\text{ cm.}}{280\text{ m}\mu}$	
DEAE cellulose-frontal	serum	—	—
Hydroxylapatite-frontal	crudest	0.008	18
Hydroxylapatite-gradient	crude	0.020	45
DEAE - gradient	Fr. I	0.026	59
DEAE - gradient	Fr. II	0.020	45
—	Fr. I *	0.042	95
—	Fr. II *	0.032	73

Method for the fractionation, concentration, and purification of human ceruloplasmins from whole serum: the purity of the ceruloplasmin preparation obtained at each step is determined from the ratio of blue color ($\text{OD}_{610\text{ m}\mu}^{1\text{ cm.}}$) to total protein ($\text{OD}_{280\text{ m}\mu}^{1\text{ cm.}}$) which is 0.044 for a solution of pure ceruloplasmin.

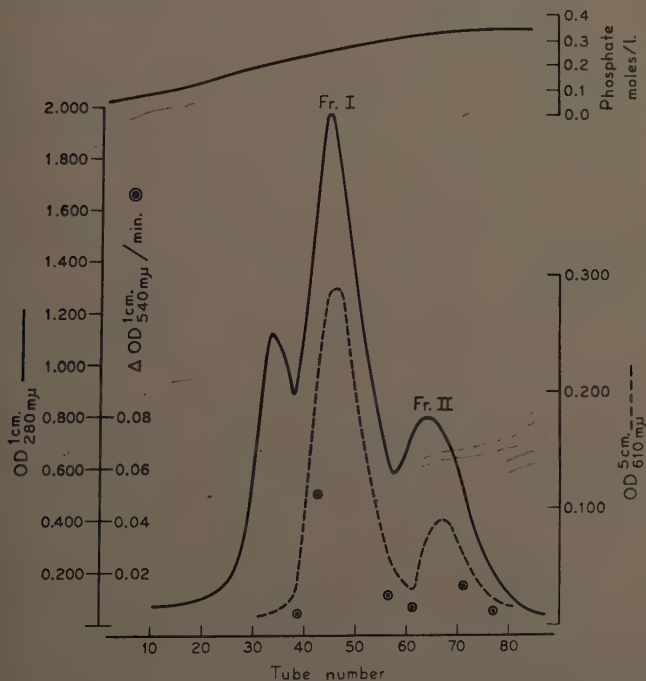


FIGURE 1. Gradient elution chromatography of crude ceruloplasmin, prepared from the pooled serum of 10 normal adult donors, on a hydroxylapatite column. The height of the column was 16.2 cm. and the internal diameter was 1.8 cm. The constant volume mixing chamber contained 200 cc. of 0.1 *M* phosphate buffer of pH 6.4. The addition flask contained a 0.45 *M* phosphate buffer of the same pH. In FIGURES 1 to 5 inclusive, total protein is given by $\text{OD}_{280\text{ m}\mu}^{1\text{ cm.}}$; PPD oxidase activity, by $\Delta \text{OD}_{540\text{ m}\mu}^{1\text{ cm.}}/\text{min.}$; blue color of ceruloplasmin by $\text{OD}_{610\text{ m}\mu}^{5\text{ cm.}}$; and the concentration of the eluant by the topmost curve. In FIGURES 1, 2, and 3 the scales for total protein and blue color were selected in such a manner that the 2 respective curves would be superimposed for a solution of pure ceruloplasmin.

band, visible at the top of the column, was eluted frontally with a solution of 0.3 *M* sodium chloride in 0.05 *M* phosphate buffer of pH 6.4. A turbid yellow-green solution was obtained that was centrifuged in a Spinco Model L ultracentrifuge at 105,000 *g* for 15 min., and the clear yellow-green supernatant was decanted.

This was diluted with 5 times its volume of 0.05 *M* phosphate buffer of pH

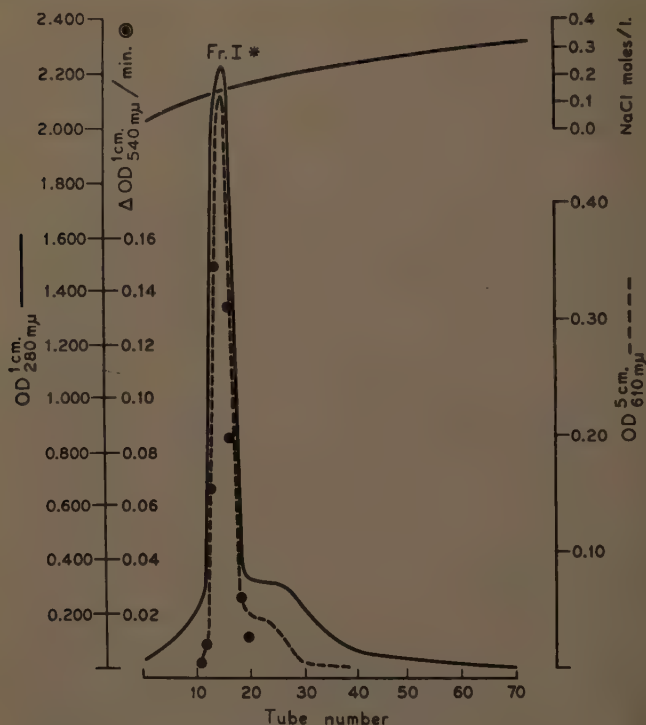


FIGURE 2. Gradient elution chromatography of fraction I ceruloplasmin obtained from the pooled serum of 10 normal adult donors, on a DEAE column, yielding purified fraction I*. Height: 12.3 cm.; internal diameter: 1.8 cm. The constant volume mixing chamber contained 100 cc. of 0.1 *M* sodium chloride in a 0.05 *M* phosphate buffer of pH 6.4. The addition flask contained 0.35 *M* sodium chloride solution made up in the same buffer.

6.4, and the ceruloplasmin was adsorbed onto a hydroxylapatite column¹² equilibrated with the same buffer. The column was then washed with the buffer and with 0.1 *M* phosphate buffer of pH 6.4 yielding a yellow material that was discarded. The blue ceruloplasmin band was eluted frontally, with a 0.4 *M* phosphate buffer of pH 6.4 yielding a crude ceruloplasmin preparation.

This was dialyzed for 24 hours against a 0.05 *M* phosphate buffer of pH 6.4. The heavy white precipitate formed in the dialysis sac was centrifuged down, and a clear blue solution was obtained. The ceruloplasmin was adsorbed onto a hydroxylapatite column at 5° C. and subjected to gradient elution (FIGURE

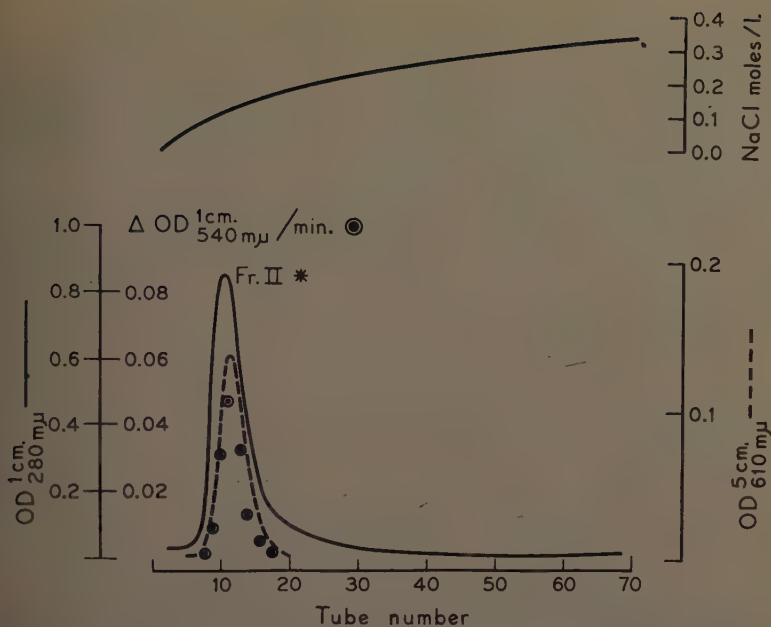


FIGURE 3. Gradient elution chromatography of fraction II ceruloplasmin obtained from the pooled serum of ten normal adult donors, on a DEAE column, yielding purified fraction II.* Height: 11 cm.; internal diameter: 0.9 cm. The gradient was made as in FIGURE 2

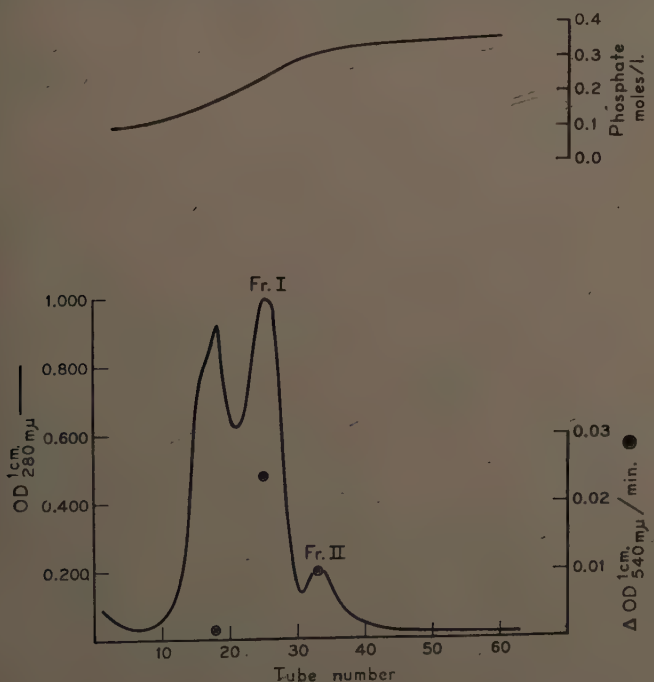


FIGURE 4. Gradient elution chromatography of crude ceruloplasmin prepared from pooled serum obtained from the cord blood of four newborn infants, on a hydroxylapatite column. Height: 7 cm.; internal diameter: 1.2 cm. The constant volume mixing chamber contained 75 cc. of 0.1 M phosphate buffer of pH 6.4. The addition flask contained 0.45 M phosphate buffer of the same pH.

1). Three-cc. samples were collected in each of 80 tubes. The PPD oxidase activity and the conductivity were determined on samples from selected tubes.

For further purification, without apparent further fractionation, all of the normal adult samples of each of the two fractions in FIGURE 1 that contained ceruloplasmin, as indicated by blue color or PPD oxidase activity, were combined. Both were concentrated by ultrafiltration through separate collodion

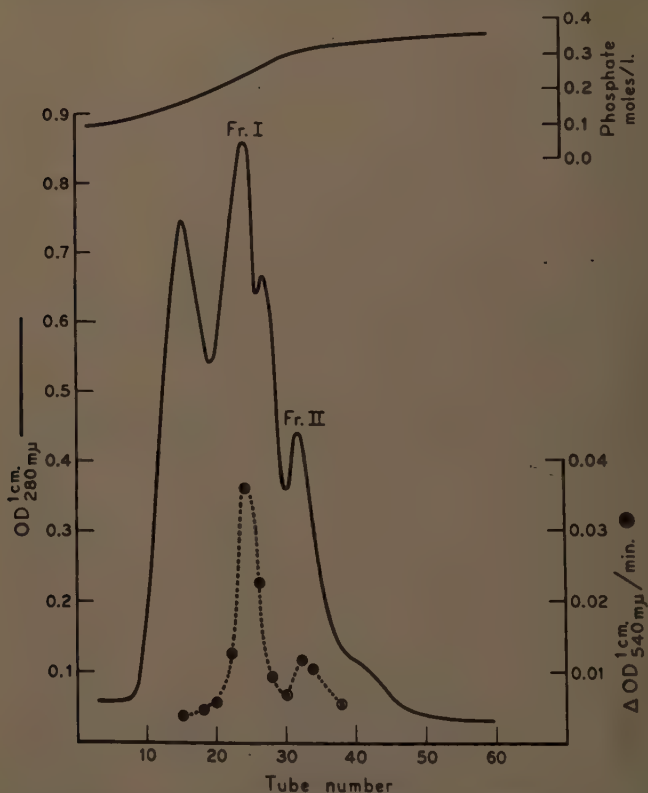


FIGURE 5. Gradient elution chromatography of crude ceruloplasmin, obtained from the serum of a patient with Wilson's disease (H. T.), on a hydroxylapatite column. The dimensions of the column and gradient were as given in FIGURE 4.

membranes. In both cases a blue gel was obtained that was dissolved in approximately 10 cc. of a 0.05 *M* phosphate buffer of *pH* 6.4 and centrifuged in the Spinco Model L ultracentrifuge at 105,000 *g* for 15 min. The two resultant clear deep blue solutions were each analyzed by gradient elution chromatography on separate DEAE columns (FIGURES 2 and 3). Samples were collected and analyzed as in the gradient elution from hydroxylapatite and appropriate ones were selected for starch-gel electrophoresis at *pH* 5.5 and *pH* 8.6⁷ after dialysis against the buffer used for making the gels.

Starch-gel electrophoreses of the serum of the newborns and the patient

with Wilson's disease were carried out on material obtained by gradient elution from hydroxylapatite (FIGURES 4 and 5) without the further purification step of gradient elution from a DEAE column.

Results

Gradient elution chromatography on a hydroxylapatite column of the crude ceruloplasmin preparations from the normal human adult (FIGURE 1), the newborn infant (FIGURE 4), and the patient with Wilson's disease (FIGURE 5) yielded 2 fractions containing ceruloplasmin. The major fraction was termed I and the minor, II. The ratio of the concentration of fraction I to that of

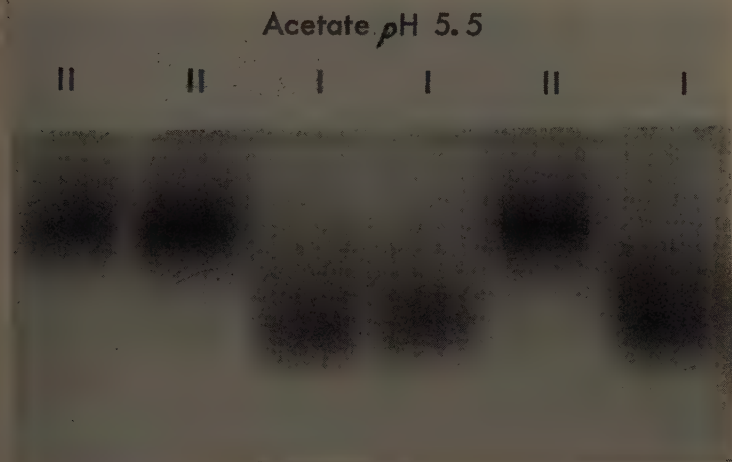


FIGURE 6. Electrophoresis at pH 5.5 on starch gel of the 2 ceruloplasmin fractions obtained from ten normal human adults. I = fraction I ceruloplasmin; II = fraction II ceruloplasmin; the migration is downward in the direction of the anode. Staining of ceruloplasmin in FIGURES 6 to 11 by means of PPD.

fraction II, as measured by blue color (FIGURE 1) or enzymatic activity (FIGURES 4 and 5), was found to be approximately 3:1 in all 3 cases.

Starch-gel electrophoresis of samples of these 2 chromatographic fractions of ceruloplasmin indicated, first, that fraction II ceruloplasmin has a lower mobility than fraction I ceruloplasmin at pH 5.5 in each type of individual (FIGURES 6, 8, and 10). This is also true at pH 8.6 (FIGURES 7, 9, and 11) although the differences in mobility between the two fractions are slight at this pH. Second, fraction II ceruloplasmin, obtained from the normal human adult, has a lower mobility at pH 5.5 than the corresponding fraction II ceruloplasmin obtained either from the newborn infants (FIGURE 8), or from the patient with Wilson's disease (FIGURE 10). This suggests that fraction II ceruloplasmin from newborn infants or from patients with Wilson's disease may be qualitatively different from fraction II ceruloplasmin of the normal human adult.

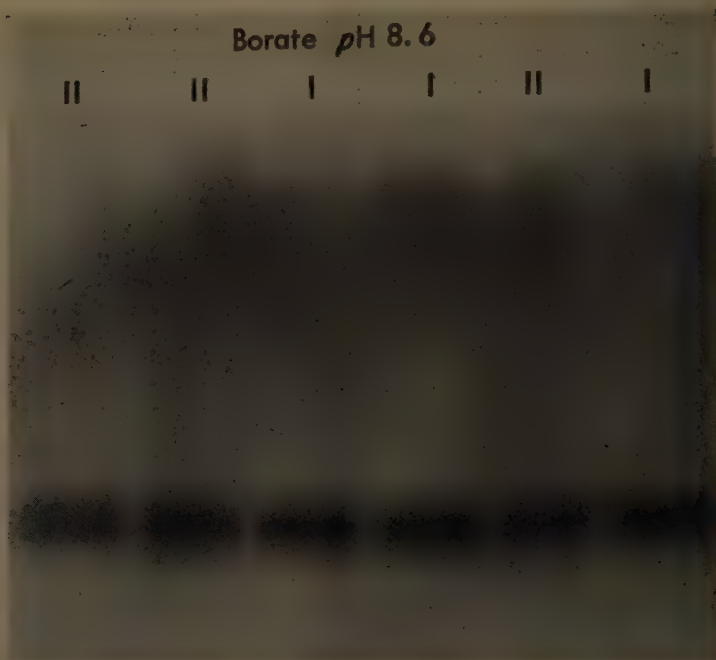


FIGURE 7. Electrophoresis at pH 8.6 on starch gel of the 2 ceruloplasmin fractions obtained from 10 normal human adults. Symbols and direction of migration as in FIGURE 6.

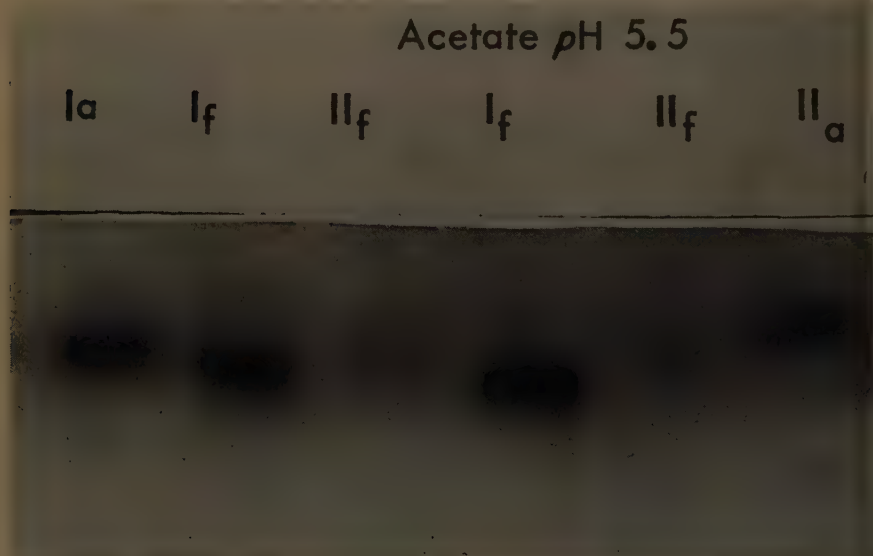


FIGURE 8. Electrophoresis at pH 5.5 on starch gel of the 2 ceruloplasmin fractions obtained from the newborn infant compared to those of the normal adult. I_a = fraction I of the normal adult. II_a = fraction II of the normal adult. I_f and II_f represent the corresponding fractions from the newborn infant. The migration is downward in the direction of the anode.

Borate, pH 8.6

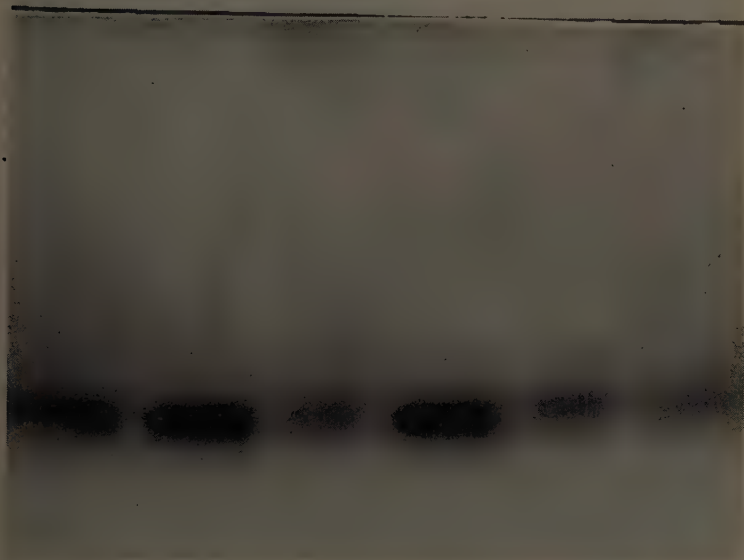
I_a I_f II_f I_f II_f II_a

FIGURE 9. Electrophoresis at pH 8.6 on starch gel of the 2 ceruloplasmin fractions obtained from the normal infant compared to those of the normal adult. Symbols and direction of migration as in FIGURE 8.

Acetate pH 5.5

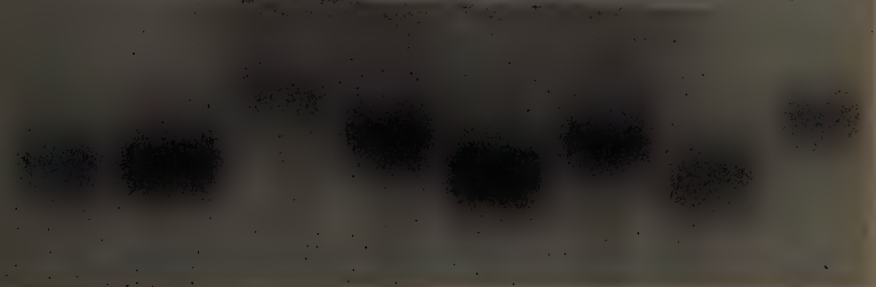
I_a I_w II_a II_w I_w II_w I_a II_a

FIGURE 10. Electrophoresis at pH 5.5 on starch gel of the 2 ceruloplasmin fractions obtained from a patient with Wilson's disease (H.T.) compared to those of the normal adult. I_a and II_a represent fraction I and fraction II, respectively, obtained from the normal human adult. I_w and II_w represent the corresponding fractions from a patient with Wilson's disease. The migration is downward in the direction of the anode.

Discussion

The significance of the heterogeneity described in this paper is unknown. We do not even know if these two types of ceruloplasmins represent genetic or acquired variants. Indeed, one may ask whether these variants are not simply artifacts of the fractionating procedure. Two of us (A.G.M. and I.H.S.) were troubled by this consideration when we found, in the previous work referred to above, four variants of ceruloplasmin⁷ in material prepared by alcohol fractionation procedures modified from those of Cohn. It was as a result of this concern that we carried out the studies we are now reporting which involve the treatment of fresh whole serum at pH values no lower than 6.4 without the addition of any soluble organic reagents. These procedures

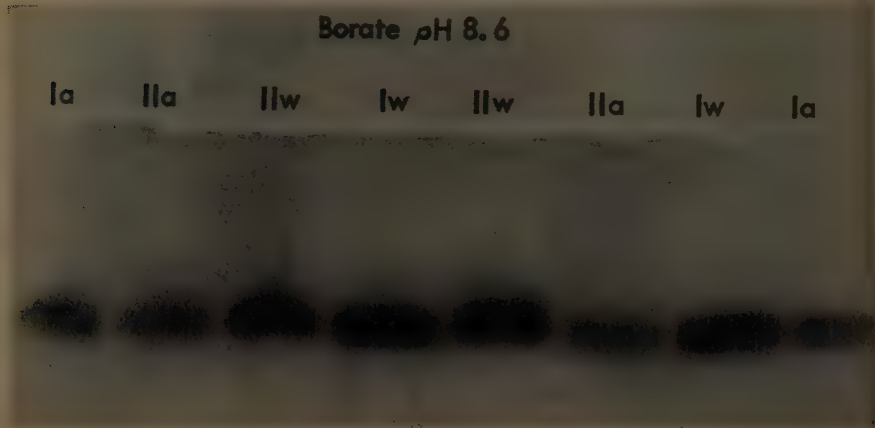


FIGURE 11. Electrophoresis at pH 8.6 on starch gel of the 2 ceruloplasmin fractions obtained from a patient with Wilson's disease (H.T.) compared to those of the normal adult. Symbols and direction of migration as in FIGURE 10.

appear to us to be of such a mild nature as not to be likely to cause changes in ceruloplasmin of the kind we have observed.

Our results are not in accord with those recently reported by Richterich,¹³ who found that, in contrast to the normal human adult, newborn infants and patients with Wilson's disease have a greater concentration of fraction II ceruloplasmin than of fraction I. We found fraction I to be the major component in all three types of individuals. Our other finding, that fraction II ceruloplasmin from newborn infants and from a patient with Wilson's disease may be qualitatively different from fraction II of the normal human adult, requires further investigation.

Summary and Conclusions

Chromatography of fresh whole serum indicates that there are two chromatographically different fractions of ceruloplasmin in normal human adults, newborn infants, and in one patient with Wilson's disease.

Despite the marked deficiency of ceruloplasmin in the neonates and in the patient with Wilson's disease, the ratio of the concentrations of the two chromatographic fractions appears to be the same in these subjects as in the normal adult.

Starch-gel electrophoresis, at pH 5.5 and pH 8.6, of the two chromatographic fractions of ceruloplasmin from these individuals demonstrated several differences in mobility between the 2 fractions.

References

1. HOLMBERG, C. G. & C. B. LAURELL. 1948. *Acta Chem. Scand.* **2**: 550.
2. SCHEINBERG, I. H. & I. STERNLIEB. 1960. *Pharmacol. Rev.* **3**: 355.
3. SCHEINBERG, I. H. & D. GITLIN. 1952. *Science*. **116**: 484.
4. BROMAN, L. 1958. *Nature*. **182**: 1655.
5. SANKAR, S. 1959. *Federation Proc.* **18**: 441.
6. URIEL, J. 1958. *Bull. Soc. Chim. Biol.* **39**: 105.
7. MORELL, A. G. & I. H. SCHEINBERG. 1960. *Science*. **131**: 930.
8. HOLMBERG, C. G. & C. B. LAURELL. 1951. *Acta Chem. Scand.* **5**: 476.
9. SCHEINBERG, I. H. & A. G. MORELL. 1957. *J. Clin. Invest.* **36**: 1193.
10. SPAHR, P. F., J. T. EDSALL, A. G. MORELL & I. H. SCHEINBERG. Unpublished observations.
11. SOBER, H. A., F. J. GUTTER, N. M. WYCKOFF & E. A. PETERSON. 1956. *J. Am. Chem. Soc.* **78**: 751.
12. TISELIUS, A., S. HJERTEN & O. LEVIN. 1956. *Arch. Biochem. Biophysics*. **65**: 132.
13. RICHTERICH, R., E. GAUTIER, H. STILLHART & E. ROSSI. 1960. *Helv. Paed. Acta*. **5**: 424.

IMMUNOCHEMICAL STUDIES OF FUNCTIONALLY SIMILAR ENZYMES*

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For many years attempts have been made to demonstrate differences among functionally similar enzymes from different tissues of the same species. Chemical, kinetic, physical, and immunochemical methods have been employed. For example, 0.8 per cent formaldehyde inhibits red-cell acid phosphatase completely, but has no effect on prostatic acid phosphatase.¹ Kaplan and his associates have characterized the lactic dehydrogenases from different tissues by the relative rates of reduction of diphosphopyridine nucleotide (DPN) and DPN analogues.² Liver phosphoprotein phosphatase of lysosomes has been shown to differ from that in the soluble phase with respect to the K_m values obtained with α -casein and β -casein, as well as on the basis of the ratios of V_{max} . α -casein: V_{max} . β -casein.³ Several zones of lactic dehydrogenase activity in human serum were demonstrated electrophoretically in 1957 by Vesell and Bearn; the relative amounts of lactic dehydrogenase in the zones were changed in myocardial infarction and in leukemia.⁴

In 1943, Kubowitz and Ott⁵ attempted to differentiate enzymes from normal and tumor tissues in a single species by immunochemical techniques. They found that the lactic dehydrogenase isolated from either rat muscle or Jensen sarcoma of the rat was inhibited equally by rooster antiserum to the rat-muscle enzyme, and that the inhibition by the antiserum was competitive with the substrate, pyruvate. Henion and Sutherland⁶ showed that rooster antisera to dog-liver and dog-heart phosphorylases most powerfully inhibited the homologous enzyme in each case, and had less effect upon the phosphorylases from other dog tissues, as well as from heart and liver of other species. McGeachin and Reynolds^{7,8} have recently reported that antisera produced in rabbits, roosters, or rats against hog pancreatic α -amylase inhibited hog sali-

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vary, pancreatic, spleen, and serum amylases, but had no significant effect upon the α -amylase isolated from hog liver.

About 1952 a program was initiated in our laboratory at Sloan-Kettering Institute to attempt the immunochemical differentiation of functionally similar enzymes from different tissues of the same organism. The three enzymes with which we have been concerned so far have been alkaline phosphatase, phosphohexose isomerase, and lactic dehydrogenase.

Phosphohexose Isomerase

Phosphohexose isomerase (PHI) from both dog liver and human liver were studied. Rabbits were injected with liver isomerase that had been purified about 50 to 100-fold.⁹ The reaction between PHI and the rabbit antiserum involved both inhibition of the enzyme and precipitate formation in which the

TABLE 1

DISTRIBUTION OF DOG LIVER PHOSPHOHEXOSE ISOMERASE ACTIVITY FOLLOWING INCUBATION WITH AND PRECIPITATION BY ITS ANTISERUM

	Time of incubation		
	30 Min.	4 Hr.	7 Days
Activity (units) of antigen-normal rabbit-heated serum mix	10,800	10,800	8,590
Activity (units) of antigen-rabbit heated antiserum mix	8,740	8,480	6,900
Inhibition (%)	18	22	20
Isomerase activity (units) in:			
(1) Precipitate		7,585	6,810
(2) Supernatant		35	35
Recovery of enzyme in:			
(1) Precipitate (%)		89.5	98.5
(2) Supernatant (%)		0.4	0.4

precipitate retained as much as 80 per cent of the original enzymic activity (TABLE 1). At low enzyme concentration, that is, 2 μ g. of enzyme per 3.0 ml. of antiserum, up to 68 per cent of the activity of human-liver PHI was inhibited; whereas at high concentrations of enzyme, that is, 40 μ g. of enzyme per 3.0 ml. of antiserum, a maximum of only 22 per cent of the activity of dog-liver PHI was inhibited. However, all of the remaining activity could be removed by centrifugation and could be demonstrated in the resuspended precipitate.

At levels of enzyme at which dog-liver PHI was precipitated by its homologous antiserum, purified isomerases from rat liver, rabbit muscle, and rabbit erythrocytes were not significantly precipitated; however, at these same concentrations of enzyme the isomerases from dog muscle and erythrocytes were completely precipitated. Various amounts of purified preparations of PHI from human liver, human skeletal muscle, human cardiac muscle, and human erythrocytes were reacted with antiserum to human-liver PHI. At

concentrations of the antigen ranging from 1000 to about 25,000 U./ml. of incubation mixture and after incubation for 48 hours, the supernatant fluids contained a negligible fraction, 0 to 5 per cent, of the activity of the added antigen. At concentrations of 250 U./ml. of incubation mixture, the differences among the degrees of inhibition were small. Differentiation of these human-tissue isomerases was, therefore, not possible by this means. Recent work from our laboratories has indicated the presence of 1 major band of PHI in normal human serum or in homogenates of human liver by techniques of zone electrophoresis on starch granules (M. K. Schwartz, unpublished data).

Alkaline Phosphatase

Immunochemical studies of this enzyme have established the fact that antisera against dog-intestinal phosphatase completely precipitated that enzyme,

TABLE 2

PRECIPITATION OF HUMAN-INTESTINAL AND HUMAN-BONE PHOSPHATASES, SINGLY AND AS MIXTURES, BY ANTI-INTESTINAL AND ANTIBONE PHOSPHATASE SERA

Antiserum	Enzyme (μ g.)	Precipitation of activity (%)
AntiHIP-II* (0.20 ml.)	HIP, 60	86
	HBP, 286	0.7
	[HIP, 60 + HBP, 286]	104†
AntiHBP-A (1.40 ml.)	HIP, 70	4
	HBP, 340	87
	[HIP, 70 + HBP, 340]	98†

* HIP and HBP denote the phosphatases from intestine and sarcomatous bone.

† Percentage of theoretic precipitation of activity for independent behavior.

but did not precipitate the intestinal phosphatase from other mammalian species, or the phosphatases from other organs (liver, kidney) of the same species.^{10,11} Furthermore this specificity was observed even when the heterologous enzymes were tested in the presence of the dog-intestinal phosphatase. Similar studies with antisera prepared against the alkaline phosphatases from intestine and bone (osteogenic sarcomatous bone) of human origin showed that here too the antibodies were clearly capable of differentiating the enzymes from these tissues (TABLE 2).¹¹

Preliminary data, obtained with these 2 antisera, suggesting their applicability to the determination of tissues of origin of alkaline phosphatases in human serum have been extended, and are shown in TABLE 3.¹² In serum from normal subjects, 13 to 29 per cent (column 3) of the alkaline phosphatase was precipitated by antiserum to intestinal phosphatase, and about 40 to 59 per cent (column 6) by the antibodies against bone phosphatase. For serum

from cancer patients with skeletal or hepatic metastases, the corresponding ranges were 0.5 to 11 per cent and 75 to 93 per cent. Of particular note is the fact that the percentage of the total enzyme precipitated by antiserum to bone phosphatase was especially high in cases showing skeletal metastases. From these data it would appear that the major portion of serum-alkaline phosphatase is of osteogenic origin, with the intestine contributing a much smaller fraction of the total.

The results of this study prompted a number of questions, some of which have been the basis for further investigation. For example, the extent of

TABLE 3

THE PRECIPITATION OF HUMAN-SERUM PHOSPHATASE BY ANTI-INTESTINAL PHOSPHATASE AND ANTIBONE PHOSPHATASE SERA IN THE PRESENCE OF HORSE ANTIBODY

Clinical diagnosis	Serum phosphatase (K.A. units*)	Phosphatase precipitated by:			"Bone" phosphatase in serum† (%)	Ratio of "bone": intestinal phosphatase	Phosphatase not precipitated by antiHIP + antiHBP (%)
		Anti-HIP† (%)	Anti-HBP (%)	Anti-HIP + anti-HBP (%)			
Normal patient	8.4	29	67	69	40	1.4	31
Normal patient	4.7	13	60	61	48	3.8	39
Normal patient	8.4	13	72	72	59	4.6	28
Cancer patients							
Kidney (removed); liver metastasis	50	11	87	86	75	6.9	14
Breast; liver and skeletal metastasis	118	8	92	91	83	10	9
Cystic duct; liver metastasis	62	3	80	78	75	25	22
Prostate; skeletal metastasis	48	3	93	92	89	31	8
Prostate; skeletal metastasis	122	0.5	94	94	93	190	6

* King-Armstrong units.

† HIP and HBP denote the phosphatases from intestine and sarcomatous bone.

‡ "Bone" phosphatase values were obtained by subtracting the values in column 3 from those in column 5. See the text for explanation.

cross reactions of other tissue phosphatases with the antisera from intestine and bone was not known. In TABLE 4 are shown the results of precipitation of enzymatically equivalent amounts of the alkaline phosphatases of intestine, bone, kidney, and liver by anti-intestinal phosphatase serum and by anti-bone phosphatase serum in the absence and presence of the horse antibodies. Horse antibodies (horse, antirabbit, γ -globulin antibodies) were included in this study to simulate conditions of the serum systems where they were included to insure maximum precipitation of the very low levels of phosphatase present.

With the antiserum against intestinal phosphatase, 87 and 91 per cent of the intestinal enzyme was precipitated in the absence and in the presence of the horse antibodies respectively. In contrast, the enzymes from the other

organs were only slightly precipitated under these conditions. With anti-serum against bone phosphatase, the intestinal enzyme was readily differentiated from the bone enzyme in the absence of horse antibodies. On the other hand, the serological behavior of the enzymes from bone, liver, and kidney were similar, whether precipitation was carried out in the absence of horse antibodies (64 to 77 per cent precipitation) or in their presence (89 to 94 per cent precipitation). Whether kidney or liver contributes to the serum alkaline phosphatase under normal or pathological conditions is not yet known.

Evidence of an independent nature with which the immunochemical differences between the phosphatase of intestinal origin and those from the other tissues might be correlated was sought in the relative activities of the different tissue phosphatases when tested under the same conditions against several substrates (W. Landau and M. Schlamowitz, unpublished data) and in their

TABLE 4
CROSS REACTION OF ANTI-INTESTINAL AND ANTIBONE PHOSPHATASE SERA WITH
ALKALINE PHOSPHATASES FROM OTHER TISSUES

Antiserum	Enzyme (μ g.)	Precipitation of enzyme activity:	
		Without horse antibody (%)	With horse antibody (%)
AntiHIP-II* (0.20 ml.)	HIP-II 28.1	87	91
	HBP-A 87.4	0	6
	HKP-19-II 1180	2	10
	HLP-50-II 405	—	12
AntiHBP-A (0.50 ml.)	HBP-A 83.2	77	94
	HIP-II 22.0	4	61
	HKP-19-II 1180	64	89
	HLP-50-II 405	74	92

* HIP, HBP, HKP, and HLP denote the phosphatases from human intestine, bone, kidney, and liver, respectively.

chromatographic properties (A. L. Grossberg, E. S. Harris, and M. Schlamowitz, unpublished data). The results of the studies with different substrates are shown in TABLE 5. Two differences seem to stand out. The relative activities of the enzymes from bone, liver, kidney, and spleen against *p*-nitrophenylphosphate were about 83 to 104 per cent, whereas the activity of the intestinal phosphatase was only about 45 per cent of the activity with the reference substrate, β -glycerophosphate. Conversely the activity with adenosine-5'-phosphate was 131 per cent for the intestinal enzyme, but only about 53 to 67 per cent for the enzymes from the other tissues. The difference between the intestinal phosphatase and the enzymes from other tissues investigated appear thus far to be reflected both serologically and by studies of their "substrate spectra."

Chromatography of pooled preparations of enzyme from each tissue served to indicate a high degree of chromatographic homogeneity for bone, liver, and spleen phosphatases on carboxymethylcellulose (CMC) and diethylamino-

ethylcellulose (DEAE), and a heterogeneity of the enzyme preparations from intestine and kidney (FIGURE 1). The range of variation to be encountered is shown in the chromatographic analysis of individual specimens from intestine and kidney, the tissues displaying the greatest heterogeneity in the pooled samples (FIGURE 2). With intestine, there is in every case a major component with the "substrate spectrum" characteristics already described. In addition a fraction, eluting just before this main peak, appears in some cases. In one case, still another minor component (approximately 15 per cent) was eluted from the column prior to the start of the gradient; it was interesting to note that this component showed the substrate spectrum of the enzymes from liver, kidney, bone, and spleen. With kidney, 2 principal chromatographic components were seen (the latter probably heterogeneous), most often in approximately a 40:60 ratio, although in 1 case a ratio of 15:85 was found.

TABLE 5
RELATIVE ACTIVITIES OF HUMAN-TISSUE ALKALINE PHOSPHATASES
ON PHOSPHOMONOESTER SUBSTRATES

Substrate	Relative activity*					
	HIP-V†	HBP-II	HLP-IV	HSP-IV	HKP-III-A	HKP-III-B
β -Glycerophosphate	100	100	100	100	100	100
<i>p</i> -Nitrophenyl phosphate	45	103	83	100	99	104
Threonine phosphate	89	85	88	81	81	81
Phenolphthalein phosphate	47	71	82	71	69	71
Adenosine-5'-phosphate	131	66	65	57	53	67
Phosphoglyceric acid	48	23	34	30	31	31

* Activities were measured at pH 9.7, 37° C. with 0.02 *M* substrate for 20 min., during which the release of phosphate as a function of time was linear.

† Preparations designated II are obtained by fractionation with ethanol; III is preparation II chromatographed on CMC; IV is preparation III chromatographed on DEAE; and V is preparation IV rechromatographed on DEAE. The first enzyme fraction eluted from CMC is designated *A*; the second, *B*.

The substrate spectrum of individual kidney specimens resembled that of the pooled preparation (TABLE 5) and was about the same for both chromatographic fractions. Parallel chromatographic distribution of phosphatase activity was observed with each of the substrates tested.

At this point it was desirable to investigate the possible influence of the mode of preparation of the enzyme on chromatographic behavior. Two alkaline phosphatase preparations from hog kidney were prepared, one by the autolytic procedure used with the human phosphatases, and the other from fresh kidney by the butanol extraction procedure.¹³ The chromatographic behavior and substrate spectra of the 2 preparations were then compared. It may be seen from FIGURE 3 that the chromatographic behavior of the autolyzed preparation resembles that observed with human kidney phosphatase; two fractions were obtained with elution complete at about 0.125 *M* Cl⁻. This pattern was different from the butanol preparation that chromatographed predominantly as a single component eluted by about 0.40 *M* Cl⁻. However,

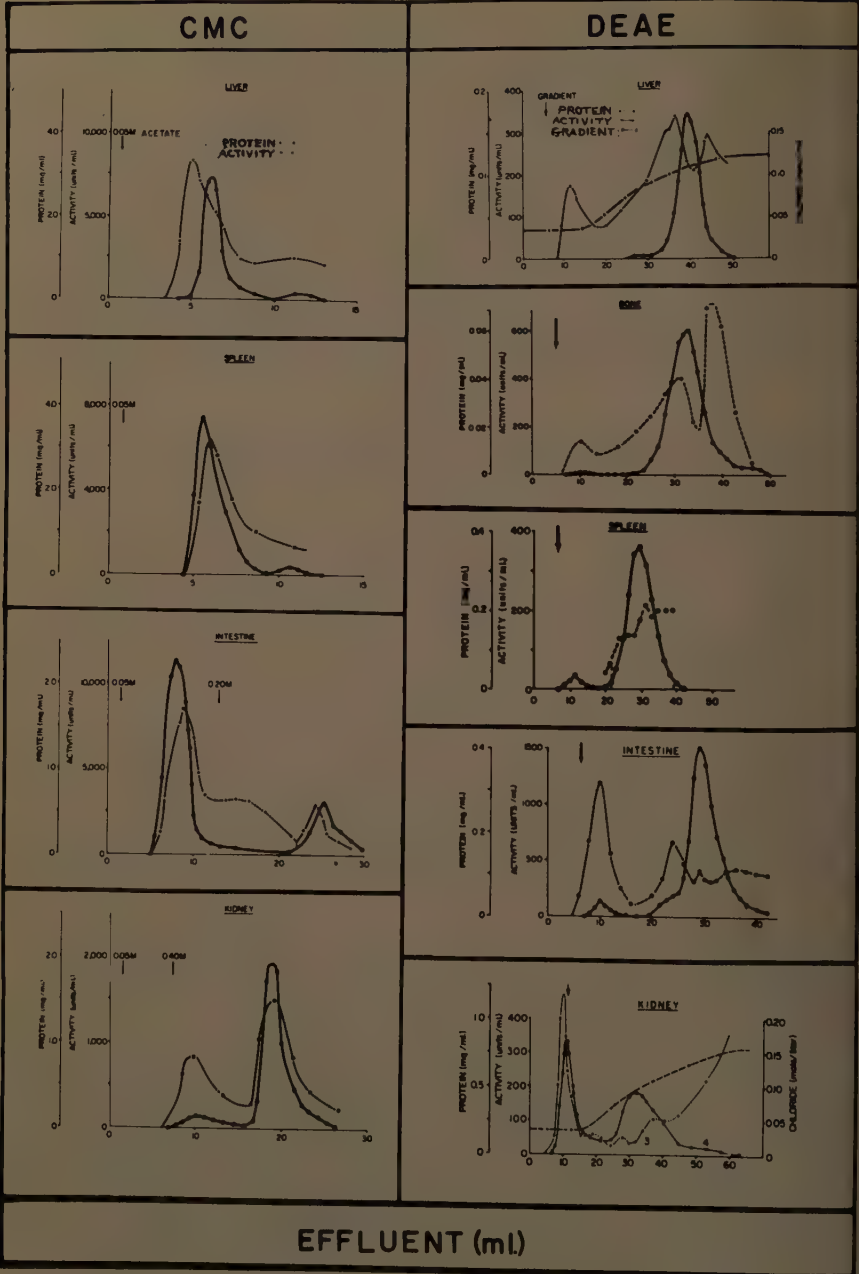


FIGURE 1. Chromatography of human tissue alkaline phosphatases on carboxymethylcellulose (CMC) and diethylaminoethylcellulose (DEAE). With DEAE cellulose, the elution gradient shown for liver applies as well for bone, spleen, and intestine.

as far as substrate spectrum was concerned there was no difference (TABLE 6); it appeared to be characteristic of the tissue.

Lactic Dehydrogenase

The activity of this enzyme, like that of other glycolytic enzymes, is increased in the serum of patients with a variety of diseases. Such elevations

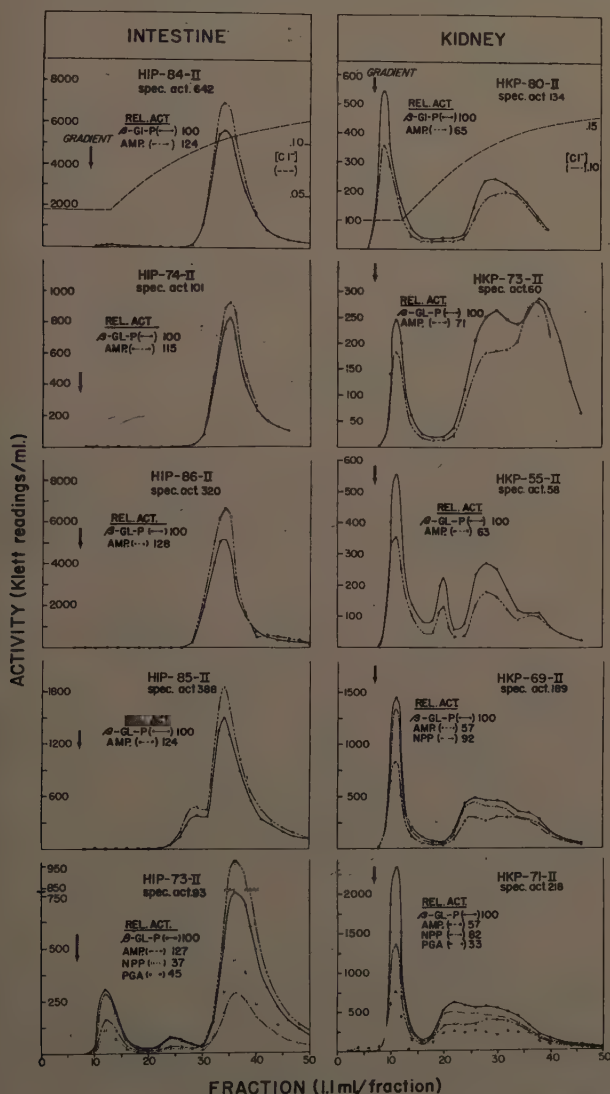


FIGURE 2. Chromatography on DEAE of alkaline phosphatase from individual specimens of human intestine and kidney. The elution gradient for each tissue phosphatase is shown in the top frame.

have been reported in patients with hepatitis,¹⁴ myocardial infarction,¹⁵ and certain types of cancer including leukemia.¹⁶

Early studies of the LDH of rabbit tissues showed the feasibility of using immunochemical methods to differentiate among the tissue LDHs.^{17,18} In studies in this laboratory it was shown that roosters treated intravenously with alum-absorbed crystalline LDH from rabbit skeletal muscle produced antibodies that, under suitable conditions, completely inhibited the activity of

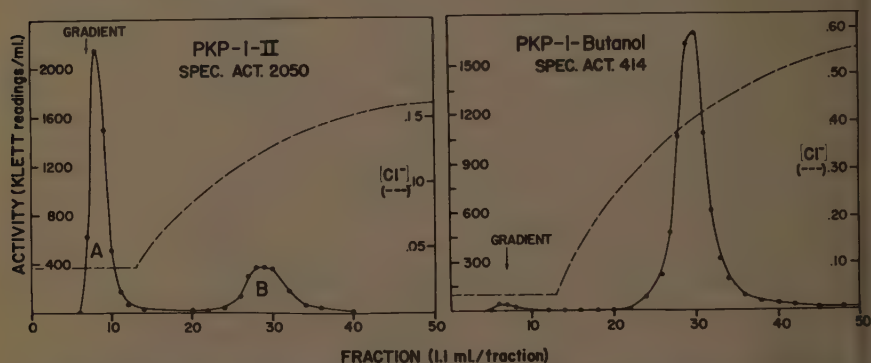


FIGURE 3. Chromatography on DEAE of porcine kidney alkaline phosphatase prepared by autolysis (*left*), and by extraction with butanol (*right*).

TABLE 6

RELATIVE ACTIVITIES ON PHOSPHOMONOESTER SUBSTRATES OF PORCINE KIDNEY PHOSPHATASES OBTAINED BY AUTOLYSIS AND BY BUTANOL EXTRACTION

Substrate	Relative activity*	
	PKP-1-II(DEAE)A†	PKP-1-Butanol
β -Glycerophosphate	100	100
p-Nitrophenylphosphate	88	91
Adenosine-5'-phosphate	61	68

* Activities were measured at pH 9.7, 37° C. with 0.02 M substrate for 20 min., during which the release of phosphate as a function of time was linear.

† PKP-1-II(DEAE)A is the first peak eluted when PKP-1-II (the ethanol fraction from autolyzed kidney, specific activity, 2050 U./mg. protein) is passed through DEAE. PKP-1-Butanol is the aqueous fraction of a butanol extract of fresh kidney, specific activity, 1035 U./mg. protein.

the homologous enzyme (FIGURE 4), that is, 50 μ g. of crystalline LDH was completely inhibited by 1 ml. of antiserum. The extent of inhibition of homologous enzyme by antiserum was found to be independent of the concentration of reactants and to depend solely upon the relative concentrations of rabbit-muscle LDH and its antiserum.¹⁷ No competition between antiserum and either pyruvate or DPN could be demonstrated. The interaction of LDH from other rabbit organs with antiserum to rabbit-muscle LDH is shown in TABLE 7. It can be seen that the LDHs from liver and skeletal muscle are strongly inhibited by the antiserum to the rabbit-muscle enzyme, while the ac-

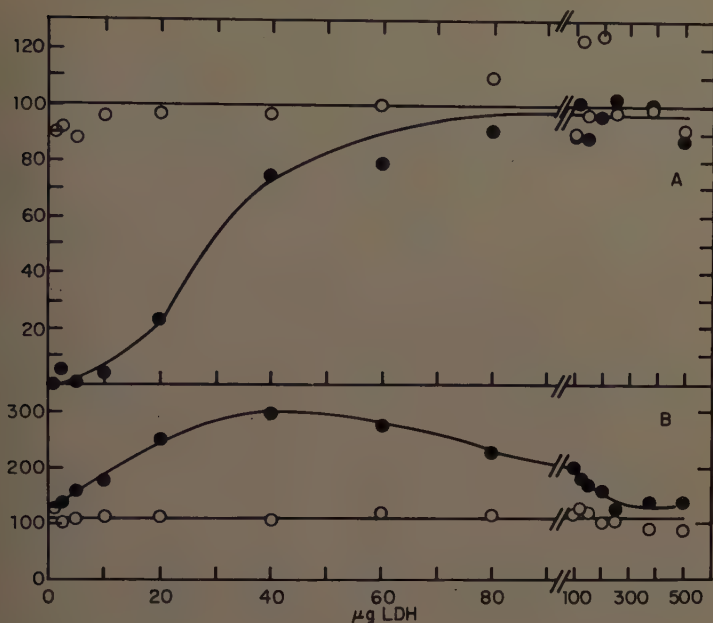


FIGURE 4. Rabbit-muscle LDH solution (0.2 ml.) was added to 0.2 ml. of normal human serum (*open circles*) or antiserum (*closed circles*), and was incubated at 4° C. for 72 hours. *A*, the ordinate represents percentage of added enzyme remaining in the supernatant; and *B*, the ordinate represents μg . of protein precipitated. Reproduced by permission of *The Journal of Biological Chemistry*.

TABLE 7
INHIBITION OF LDH FROM RABBIT ORGANS BY ANTIRABBIT-MUSCLE LDH

Enzyme source	Specific activity (U./mg. protein)	Inhibition (%)
Serum	3.6	32
	14.5	45
Kidney	1.4×10^3	25
	5.0×10^3	38
Heart	1.1×10^4	9
	2.3×10^4	8
Liver	1.7×10^3	69
	1.0×10^4	73
Muscle	1.8×10^4	81
	9.5×10^4	79
50:50 serum + muscle (single determination)	—	66
		63*

* Calculated.

tivity of the enzyme from heart is practically unaffected. Only 32 per cent of the LDH in normal rabbit serum was inhibited by antiserum to the rabbit-muscle enzyme. However, all of the added rabbit-muscle enzyme in a 50:50 mixture of serum and muscle enzyme was inhibited by the antiserum. Thus it is unlikely that normal serum contains any substance that might prevent

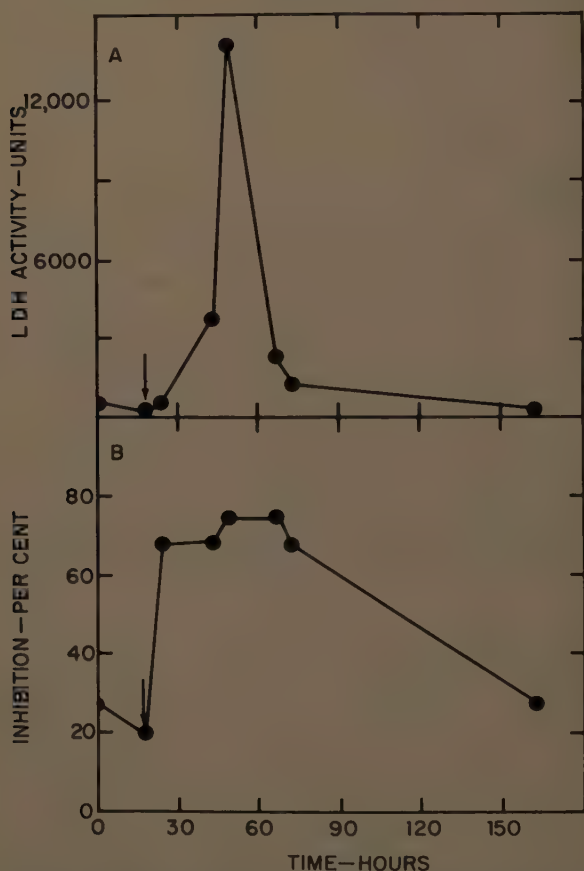


FIGURE 5. A, LDH activity of rabbit serum in response to a single dose of CCl_4 , administered at the time indicated by the arrow; and B, inhibition of rabbit-serum LDH by antiserum to rabbit-muscle LDH after CCl_4 treatment. Reproduced by permission of *The Journal of Biological Chemistry*.

the interaction of LDH with anti-LDH. TABLE 7 also shows that the interaction of LDH from rabbit organs is not influenced significantly by the state of purity of the enzyme.

FIGURE 5 illustrates the possibility of determining the organ or tissue of origin of LDH when the concentration of this enzyme is increased in rabbit serum. CCl_4 , a well-known hepatotoxic agent, was administered to rabbits at a dosage of 1.0 gm./kg. of body weight. The serum LDH level increased

40-fold, from 300 to 400 U./ml. to more than 14,000 U./ml. of serum in the CCl₄-treated animals. At the same time there was a dramatic rise in the inhibition of the serum LDH by antiserum to the muscle enzyme, from a normal value of about 25 per cent to a maximum of about 75 per cent, the same degree of inhibition obtained with liver LDH. This high level of inhibition lasted until the serum-enzyme level had returned toward normal values.

The immunochemical differentiation of LDH from human tissues was then undertaken.¹⁹ In pursuance of this goal it was considered desirable to purify, as much as possible, LDH from human tissues for use as antigens.²⁰ The schemes adopted for the purification of LDH from human heart and liver are shown in TABLE 8. In both cases the final product was a crystalline protein having a specific activity of 2.7×10^6 U./mg. of protein. For purposes of our studies, 1 U. of LDH activity was defined as that amount that caused a decrease of 0.001 absorbancy in 1 min. at 37° C. in a light path of 10 mm. in a

TABLE 8
PURIFICATION OF HUMAN LDHS

Liver		Heart	
Treatment	Specific activity (KU/mg.)	Treatment	Specific activity (KU/mg.)
Extraction	10-20	Extraction	10-20
Ca ₃ (PO ₄) ₂	58	Ca ₃ (PO ₄) ₂	62
(NH ₄) ₂ SO ₄ ; pH 5.2	150	(NH ₄) ₂ SO ₄	130
(NH ₄) ₂ SO ₄ ; pH 8.6	220	Acetone	277
Ethanol	460	(NH ₄) ₂ SO ₄	695
Chromatography; (NH ₄) ₂ SO ₄		Acetone	1,000
Rechromatography; (NH ₄) ₂ SO ₄	2,100	(NH ₄) ₂ SO ₄	1,900
	2,700	Chromatography; (NH ₄) ₂ SO ₄	2,700

reaction mixture containing a final concentration of 0.001 *M* pyruvate, 0.085 *mM* DPNH, and 0.067 *M* phosphate buffer, pH 7.4. The final volume was 3.0 ml., and contained enzyme in a suitable concentration.

Electrophoretic analysis of crystalline heart LDH at pH 8.6 in 0.1 μ veronal buffer is shown in FIGURE 6. The main component had a mobility between that of an α_1 -globulin and albumin 5.7×10^{-5} cm. v^{-1} sec.⁻¹, and comprised 91 per cent of the total. This is in accord with the observation of Vesell and Bearn⁴ that an LDH with a mobility between α_1 -globulin and albumin is increased in the serum of patients with myocardial infarction. Ultracentrifugal analysis of crystalline human heart LDH in 0.2 *M* NaCl containing 0.01 *M* phosphate buffer pH 7.4 showed this enzyme to be monodispersed at all concentrations tested between 1.57 and 0.19 per cent (FIGURE 7). The sedimentation constant extrapolated to infinite dilution was $S_{20w} = 7.6 \times 10^{-13}$ sec. From this value and from the diffusion constant determined by sedimentation equilibrium according to Ehrenberg,²¹ a molecular weight of $140,000 \pm 4,000$ was calculated.

Crystalline human-liver LDH, when subjected to electrophoresis at pH

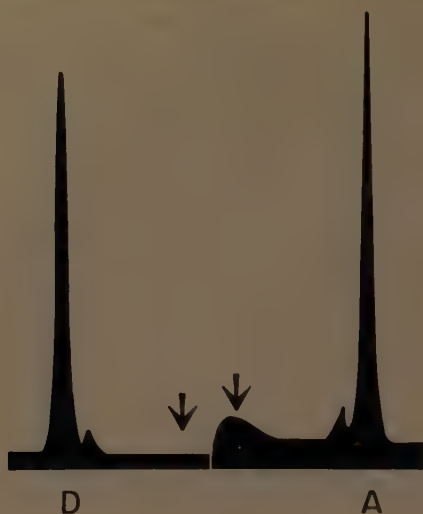


FIGURE 6. Electrophoretic pattern of purified human-heart LDH at pH 8.6 in veronal buffer, $\Gamma/2 = 0.1$. A and D are ascending and descending patterns respectively. The enzyme had a specific activity of 2.7×10^6 U. per milligram of protein.

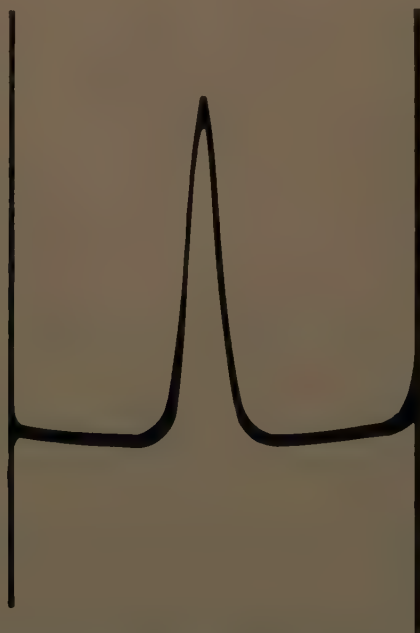


FIGURE 7. Ultracentrifuge schlieren picture of purified human-heart LDH, 1.5 per cent solution in $0.2 M$ NaCl- $0.01 M$ phosphate, pH 7.4, after sedimentation for 47 min. at 59,780 rpm.

8.6 in 0.1 μ veronal buffer, lost activity. In 1 experiment, it appeared to decompose into 2 components, 1 of which migrated rapidly toward the anode while the other precipitated in the cathode limb of the Tiselius cell. Neither of these components showed any enzymic activity. At pH 5.65 the liver LDH migrated as a single peak toward the cathode and remained fully active.

The extinction coefficient, $E_{280}^{1\% \text{ per cent}}$, of the crystalline enzyme from heart was 16.4.²⁰ The ultraviolet absorption spectrum of the enzyme from heart exhibited a shoulder at 290 $m\mu$ to 292 $m\mu$, which was not present in the enzyme from liver. The use of Goodwin and Morton's formula²² yielded values of 0.14×10^{-3} moles of tyrosine and 0.220×10^{-3} moles of tryptophan per gram of heart LDH, giving a tyrosine:tryptophan ratio of 0.63.

The pH-activity relationship of LDH from human liver and heart were the same; optimal activity was obtained from pH 7 to pH 8 for the reaction in the direction of the reduction of pyruvate. Purified liver LDH was less stable than purified heart LDH at pH values over 9.0.

Antiserum to human-liver LDH was prepared in rabbits by the intravenous administration of 28 mg. of alum-absorbed crystalline enzyme. The enzyme was given in graded doses on alternate days according to the following schedule: 4 doses each of 0.5, 1.0, 1.5, and 2.0 mg., and 2 doses of 4.0 mg. The animals were exsanguinated 1 week after the last dose, and the serum was collected and stored frozen in small batches. All 3 rabbits yielded antisera of about the same potency.

Of 8 rabbits treated with heart LDH only 1 produced any detectable antibody to the enzyme, and it was of too low a potency to be of any use. Accordingly white leghorn roosters were used; they were treated with crystalline human-heart LDH according to the schedule shown above. LDH inhibiting antibodies were produced in varying amounts. One antiserum preparation was used in all of the experiments described in this paper. Rooster blood was collected in cold heparinized tubes; the plasma was separated and stored frozen in small batches. Fibrin, which precipitated gradually from the rooster plasma, was removed before use. Neither normal rabbit nor normal rooster serum had any inhibiting or enhancing effect on the activity of human LDH. Inhibition of LDH activity was calculated from controls in which antiserum was substituted by buffered human-serum albumin, 0.15 per cent in 0.033 M phosphate buffer, pH 7.4. The LDH activity in the antiserum preparations could not be destroyed by heating to 56° C. for 30 min., and was taken into account in calculating inhibition values. Two-tenths milliliter of antiliver LDH inhibited completely 1.0 μ g. of liver LDH in a total volume of 0.4 ml.; 0.2 ml. of antiheart LDH inhibited 90 per cent of the activity of 1.0 μ g. of crystalline heart LDH in a volume of 0.4 ml. The same order of inhibition was obtained with rooster antiserum in 0.067 M phosphate buffer, 0.15 M NaCl, and 1.33 M NaCl. The latter conditions, which were reported by Goodman *et al.*²³ and Banovitz *et al.*²⁴ to be optimal for the precipitation of rooster antibodies from rooster antiserum, appeared not to influence the extent of the inhibition reaction.

Maximum inhibition in a homologous system was attained in 24 hours,

whether the reaction was allowed to proceed in concentrated reaction mixture, that is, 1.0 μ g. of enzyme and 0.2 ml. of homologous antiserum in a reaction volume of 0.4 ml., or in a 40-fold diluted reaction mixture.^{17,19} It has also been shown that the cross reaction between the antiserum to heart LDH and liver LDH was greatly reduced in diluted reaction mixtures, while the homologous reaction was not affected.¹⁹ Inhibitions by antisera to human-heart LDH and human-liver LDH of LDHs extracted from a variety of human tissues were therefore studied in diluted reaction mixtures. One ml. of tissue extract containing 200 U. of LDH activity was mixed with 1.0 ml. of a 1:20 dilution of antiserum and incubated for 2 to 4 hours at 37° C. and 65 hours at 5° C. After centrifugation at 800 g for 10 min., the activity remaining

TABLE 9
COMPARISON OF INHIBITIONS OF HUMAN TISSUE LDHs BY ANTISERA TO HUMAN-HEART AND LIVER LDHs*

LDH source	With antiserum to:	
	Liver LDH (inhibition %)	Heart LDH (inhibition %)
Heart	2	60
Crystallized heart	14	75
Liver	60	11
Crystallized liver	75	21
Lung	24	37
Spleen	22	34
Prostate	22	56
Skeletal muscle	51	16
Testes	16	34
Bone	21	49
Kidney	15	55
Brain	8	57
Erythrocytes	0	58

* Enzyme solution (1.0 ml.) containing about 200 U. of LDH was mixed with 1.0 ml. of 1:20 diluted antiserum. After incubation for 2 to 4 hours at 37° C. and 65 hours at 5° C., the mixtures were centrifuged, and the enzyme activity of 0.5 ml. of the clear supernatant was determined.

in the supernatants was determined and compared with controls in which antiserum had been substituted by albumin. The results (TABLE 9) show that antiliver LDH significantly inhibited the activity of LDH from liver and skeletal muscle, but had little effect upon the activity of LDH from the other tissues tested, including heart. Antiheart LDH, on the other hand, inhibited LDH from heart, prostate, bone, kidney, and erythrocytes, and had little effect on the activity of the enzyme from liver and skeletal muscle. When crystalline LDH from either liver or heart was added to normal human serum the homologous antiserum inhibited most of the activity of the added enzyme, while the heterologous reaction was minimal (TABLE 10).

Preliminary studies on 36 samples of serum from 14 presumably normal individuals gave an average LDH activity of 30 ± 4.6 U./ml. of serum. The average inhibition of normal serum LDH by the antibody to liver LDH was

26 \pm 7 per cent, and the inhibition by antiserum to the heart enzyme was 60 \pm 8 per cent. In the serum of patients with hepatitis the LDH level was moderately elevated. The percentage inhibition of the serum enzyme by antiliver LDH was significantly increased, while the inhibition by antiheart

TABLE 10
INHIBITION OF LDH IN HUMAN SERUM BY ANTISERA TO
HEART AND LIVER LDHS*

Serum	Activity (U.)	With antiserum to:			
		Liver LDH		Heart LDH	
		Inhibition (%)	Inhibition of added LDH (%)	Inhibition (%)	Inhibition of added LDH (%)
Normal	36	31	—	53	—
Fortified with heart LDH	133	16	10	72	79
Fortified with liver LDH	155	67	78	16	5

* Equal volumes of 10-fold diluted serum or fortified serum and 20-fold diluted antiserum were mixed and incubated for 2 hours at 37° C. and 66 hours at 5° C. After centrifugation the LDH activity of the clear supernatant was determined. Activities are expressed per 1.0 ml. of incubation mixture, and are equivalent to the units used in assaying serum LDH activities.

TABLE 11
INHIBITION OF LDH IN THE SERUM OF A PATIENT WITH HEPATITIS*

Date	Activity (U./ml.)	Inhibition with antiserum to:	
		Liver (%)	Heart (%)
7-13-60	78	51	28
7-16-60	62	54	34
7-19-60	68	61	37
7-22-60	66	55	29
7-27-60	62	61	37
7-29-60	64	54	23
8-17-60	30	29	48
8-19-60	30	36	57
9-19-60	30	22	53
10-19-60	34	29	58

* Date of infection not known.

LDH was markedly lower. This reversal of the normal pattern persisted as long as the serum LDH level was elevated. These features are illustrated in TABLE 11.

Discussion

In our immunochemical studies of functionally similar enzymes three types of reactions have been encountered. Alkaline phosphatase was precipitated

in fully active form by its antiserum. Phosphohexose isomerase was partially inhibited by its antiserum, and the remainder of the activity was manifest in suspensions of the precipitate. Lactic dehydrogenase was inhibited by antiserum under conditions that precipitated the enzyme and also in dilute reaction mixtures where no precipitate formation was detectable. The precipitated enzyme-antibody complex showed no enzymic activity under a variety of conditions.¹⁷ All three types of enzyme-antienzyme interactions have been reported by other workers. Precipitates of tyrosinase²⁵ and its antibody exhibited full enzymic activity. Urease-antiurease systems²⁶ behaved similarly to the PHI-antiPHI system in that, under conditions that precipitated all of the enzyme, the suspended precipitates retained from 20 to 70 per cent of the original urease activity. The amount of enzymic activity retained in these precipitates was shown to be dependent upon the antibody:antigen ratio, and not upon the stage of aggregation of the precipitated complex. Amylase,⁷ carboxypeptidase,²⁷ phosphorylase,⁶ and ribonuclease,²⁸ in combination with specific antibodies, all formed precipitates that were enzymically inactive when tested in suspension.

Our studies, both by immunochemical and electrophoretic techniques, indicate the absence of any sharp intraspecies tissue differences in the case of PHI. The difference between human-bone and human-intestinal alkaline phosphatase, which was immunochemically demonstrated, has also been noted with regard to inhibition by bile acids²⁹ and amino acids.³⁰ In the work cited in this paper, this difference was also reflected in the different relative activities with *p*-nitrophenyl phosphate and adenosine-5'-phosphate. In differentiation, both by immunochemical means and by substrate specificity, the enzyme from bone was similar to the alkaline phosphatases from all other human tissues tested, except that from intestine. Chromatography on columns of DEAE cellulose and on carboxymethyl cellulose revealed differences even among the other tissue phosphatases. However, the chromatographic pattern of swine-kidney alkaline phosphatase is affected markedly by the method of isolation of the enzyme. Furthermore the chromatographic pattern of butanol-extracted swine-kidney alkaline phosphatase changed upon aging, or upon incubation with kidney homogenate (Schlamowitz, unpublished data). The latter experiments raise the possibility that the obtaining of multiple chromatographic or electrophoretic variants of enzymes, sometimes called "isozymes," from a single tissue may reflect the manner of treating the tissue or of isolating the enzyme.

Acknowledgments

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References

1. ABDUL-FADI, M. A. M. & E. J. KING. 1949. Properties of the acid phosphatases of erythrocytes and of the human prostate gland. *Biochem. J.* **45**: 51.
2. KAPLAN, N. O., M. M. CIOTTI, M. HAMOLSKY & R. E. BIEBER. 1960. Molecular heterogeneity and evolution of enzymes. *Science*. **131**: 392.
3. PAIGEN, K. & S. K. GRIFFETHS. 1959. The intracellular localization of phosphoprotein phosphatase activity. *J. Biol. Chem.* **234**: 299.

4. VESELL, E. S. & A. G. BEARN. 1957. Localization of lactic dehydrogenase activity in serum fractions. *Proc. Soc. Exptl. Biol. Med.* **94**: 96.
5. KUBOWITZ, F. & P. OTT. 1943. Isolierung und Kristallisation eines Gärungsfermentes aus Tumoren. *Biochem. Z.* **314**: 94.
6. HENION, W. F. & E. W. SUTHERLAND. 1957. Immunological differences of phosphorylases. *J. Biol. Chem.* **224**: 477.
7. McGEACHIN, R. L. & J. M. REYNOLDS. 1959. Differences in mammalian amylases demonstrated by enzyme inhibition with specific antisera. *J. Biol. Chem.* **234**: 1456.
8. McGEACHIN, R. L. & J. M. REYNOLDS. 1960. Inhibition of amylases by rooster antisera to hog pancreatic amylase. *Biochim. et Biophys. Acta.* **39**: 531.
9. LIPSETT, M. N., R. B. REISBERG & O. BODANSKY. 1959. Reactions of purified tissue phosphohexose isomerases with liver phosphohexose isomerase antiserum. *Arch. Biochem. Biophys.* **84**: 171.
10. SCHLAMOWITZ, M. 1954. Specificity of dog intestinal phosphatase antiserum. *J. Biol. Chem.* **206**: 369.
11. SCHLAMOWITZ, M. 1958. Immunochemical studies on alkaline phosphatases. *Ann. N.Y. Acad. Sci.* **75**(1): 373.
12. SCHLAMOWITZ, M. & O. BODANSKY. 1959. Tissue sources of human serum alkaline phosphatase as determined by immunochemical procedures. *J. Biol. Chem.* **234**: 1433.
13. MORTON, R. K. 1954. The purification of alkaline phosphatases of animal tissues. *Biochem. J.* **57**: 595.
14. BODANSKY, O., S. KRUGMAN, R. WARD, M. K. SCHWARTZ, J. P. GILES & A. M. JACOBS. 1959. Infectious hepatitis. Correlation of clinical and laboratory findings, including serum enzyme changes. *A.M.A. J. Diseases Children.* **98**: 166.
15. WRÓBLEWSKI, F., P. RUEGSEGGER & J. S. LADUE. 1956. Serum lactic dehydrogenase in acute transmural myocardial infarction. *Science.* **123**: 1122.
16. BIERMAN, H. R., B. R. HILL, L. REINHARDT & E. EMORY. 1957. Correlation of serum lactic dehydrogenase activity with the clinical status of patients with cancer, lymphomas and the leukemias. *Cancer Research.* **17**: 660.
17. NISSELBAUM, J. S. & O. BODANSKY. 1959. Reactions of lactic dehydrogenase from various rabbit organs with anti-rabbit muscle lactic dehydrogenase. *J. Biol. Chem.* **234**: 3276.
18. GREGORY, K. F. & F. WRÓBLEWSKI. 1958. Preparation and properties of purified antilactic dehydrogenase. *J. Immunol.* **81**: 359.
19. NISSELBAUM, J. S. & O. BODANSKY. 1961. Reactions of human tissue lactic dehydrogenases with antisera to human heart and liver lactic dehydrogenases. *J. Biol. Chem.* **236**: 401.
20. NISSELBAUM, J. S. & O. BODANSKY. 1961. Purification and properties of human heart lactic dehydrogenase. *J. Biol. Chem.* **236**: 323.
21. EHRENBERG, A. 1957. Determination of molecular weights and diffusion coefficients in the ultracentrifuge. *Acta Chem. Scand.* **11**: 1257.
22. BEAVER, G. H. & E. R. HALIDAY. 1952. *In Advances in Protein Chemistry.* **VII**: 375. M. L. Anson, K. Baily and J. T. Edsall, Eds. Academic Press. New York, N.Y.
23. GOODMAN, M., H. R. WOLFE & S. NORTON. 1951. Precipitin production in chickens. VI. The effect of varying concentrations of NaCl on precipitate formation. *J. Immunol.* **66**: 225.
24. BANOVITZ, J., S. J. SINGER & H. R. WOLFE. 1959. Precipitin formation in chickens. XVIII. Physical chemical studies on the complexes of bovine serum albumin and its chicken antibodies. *J. Immunol.* **82**: 481: 489.
25. ADAMS, M. H. 1942. The reaction between the enzyme tyrosinase and its specific antibody. *J. Exptl. Med.* **76**: 175.
26. MARUCHI, M. M. & A. A. MAYER. 1955. Quantitative studies on the inhibition of crystalline urease by rabbit antiurease. *Arch. Biochem. Biophys.* **54**: 330.
27. SMITH, E. L., B. V. JAGER, R. LUMRY & R. R. GLANZ. 1952. Precipitation and inhibition of carboxypeptidase by specific antisera. *J. Biol. Chem.* **199**: 789.
28. BROWN, R. K., R. DELANEY, L. LEVINE & H. VAN VUNAKIS. 1959. Studies on the antigenic structure of ribonuclease. I. General role of hydrogen and disulfide bonds. *J. Biol. Chem.* **234**: 2043.
29. BODANSKY, O. 1937. Are the phosphatases of bone, kidney, intestine, and serum identical? The use of bile acids in their differentiation. *J. Biol. Chem.* **118**: 341.
30. BODANSKY, O. 1948. The inhibitory effects of DL-alanine, L-glutamic acid, L-lysine, and L-histidine on the activity of intestinal, bone and kidney phosphatases. *J. Biol. Chem.* **174**: 465.

HETEROGENEITY OF LACTIC AND MALIC DEHYDROGENASE IN SERUM, CEREBROSPINAL FLUID, AND BRAIN EXTRACTS IN MAN AND SHEEP*

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Recent research has drawn attention to the fact that many enzymes can be split by electrophoretic and chromatographic methods into several fractions.

It has been thus proved that the lactic acid dehydrogenase (LDH) contains five fractions. It has also been shown that the LDH content of the serum or the cerebrospinal fluid (CSF) might be increased in certain neurological diseases. We succeeded in applying the method of enzymoelectrophoresis, evolved by Wieme (1959), to neurological material. We believe it to be of interest to study the distribution of LDH in serum, CSF, and central nervous system from neurological cases in man, as well as in animals.

Previously we published the results obtained by this method for CSF. We shall not only review these findings but also give the first results obtained with the serum and especially stress the interesting possibilities offered by applying this technique to the study of the hydrosoluble proteins of the central nervous system.

Methods

The serum proteins have been first submitted to electrophoresis in agar (Wieme, 1959) and afterwards to the study of their enzymatic activity (Wieme, 1959), for LDH and malic acid dehydrogenase (MDH). The substrate used for the LDH is Na pyruvate and for MDH sodium oxalacetate.

Serum was applied without further preparation, but the CSF had to be concentrated first. The method of concentration is carried out by ultrafiltration under 10 atmospheres of nitrogen, with the apparatus of the filter *Membran Gesellschaft* from Göttingen, West Germany. We used this apparatus for several years for the study of the CSF proteins, and we carried out several thousands of concentrations with this method. Two to 4 ml. of CSF are necessary. The concentration is effected at laboratory temperature and is completed within 2 hours.

The hydrosoluble proteins of the central nervous system were extracted in an isotonic solution of saccharose. The mixture thus obtained, after having been homogenized, was centrifuged at 14,000 g at a temperature of 0° C. The supernatant fluid was then subjected to electrophoresis.

For serum and CSF as well as for cerebral proteins we always assessed the content in total LDH before effecting enzymoelectrophoresis.

For serum, CSF and cerebral extracts from sheep, we use the same technique.

After enzymoelectrophoresis was completed, we established the relative concentrations of the different enzymatic activity peaks. The relative mobility

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(m_r) of each one of these fractions was calculated by comparison with the albumin-siderophilin-dextran solution used as standard (Lowenthal *et al.*, 1960).

Results

IN MAN

In most neurological cases serum appeared to give normal results. In muscular dystrophy, where total serum LDH and MDH are increased, the distribution of the different fractions, their concentration and their m_r are normal, the enzymatic curves are more easily obtained than with normal serum (FIGURE 1).

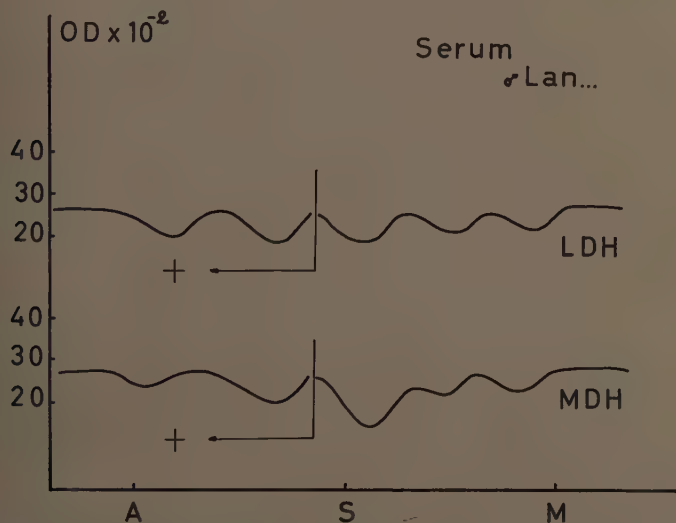


FIGURE 1. Lactic dehydrogenase and malic dehydrogenase enzymatic activity curves in serum of infantile muscular dystrophy.

The Cerebrospinal Fluid

Lactic dehydrogenase. In the CSF the LDH curves are more easily obtained than in the serum (FIGURE 2). For each of the identified fractions we have determined the concentrations and the m_r . In the serum, the most important fraction is the second; in the CSF the first (TABLE 1). The m_r of the different fractions are identical in serum and in CSF (TABLE 2).

The m_r of some of the CSF, LDH, and protein fractions are identical. This is particularly striking for the second fraction of the LDH and the α -2 globulin. On the other hand, some other fractions, more especially the first fraction of the LDH, the most important in the CSF, have m_r which does not correspond with the m_r of any particular protein. The presence of certain proteins in the CSF seems not to be detectable by the amido-schwarz. The enzymeoelectrophoresis here enables one to bring to light the presence of unsuspected protein fractions.

The m_r of the different fractions of the LDH are extremely constant. We

have been able to compare fluids of different origins, fluids which sometimes had been kept for very long periods of time, and we have always found the same m_r for the different fractions of the LDH.

Malic dehydrogenase. As we have studied the LDH, we have been able to study the MDH. Here the curve is equally easily obtained in the CSF (FIGURE 2) but it is totally different from that of the LDH. Six fractions can be identified. The most important is the fourth (TABLE 3). A very rapid fraction migrates on the level of the prealbumines. The second and third fractions of the MDH have exactly the same m_r as the first and second fractions of the LDH (TABLE 4). We wondered if this identity of m_r corresponded to reality

TABLE 3
RELATIVE CONCENTRATIONS* AND RELATIVE MOBILITIES OF THE DIFFERENT
MALIC ACID DEHYDROGENASE ACTIVITY PEAKS IN 40 SAMPLES

CSF	I	II	III	IV	V	VI
Per cent	12.1	12.5	12.9	66.9	9.3	11.2
No. of cases	27	37	39	40	4	3
m_r	1.067	0.905	0.681	0.404	0.255	0.029

Relative Mobilities of CSF Proteins (84 samples)

1.262	1.005	0.878	0.819	0.682	0.653	0.472	0.348	0.225	0.142	0.055
ρ	A	α_1	α_1'	α_2	α_2'	β_1	τ	γ_1	γ_2	γ_3

globulins

* Expressed in per cent.

TABLE 4
RELATIVE MOBILITIES OF THE DIFFERENT LACTIC AND MALIC ACID
DEHYDROGENASE ACTIVITY PEAKS IN THE CEREBROSPINAL FLUID

LDH		0.910	0.677	0.456		0.241	0.073
MDH	1.067	0.905	0.681		0.404	0.255	0.029

or whether it might be due to an artifact. For more precision, we have replaced the substrate used for determining the MDH activity (Na oxalacetate) by a mixture of asparagine and ketoglucerate, which produces in the presence of transaminase, oxalacetate. We hope to avoid in this way a possible spontaneous decarboxylation of the oxalacetate into pyruvate, which could have been the explanation for the identity between the m_r of the second and third fractions of the MDH and of the first and the second fractions of the LDH. However, after thus producing the oxalacetate extemporaneously, we get exactly the same curves, and the m_r of the different fractions are similar to what we had registered previously. The identity between the m_r of certain fractions of the LDH and of the MDH therefore is not caused by an artifact, but by the fact that certain protein fractions may present several enzymatic activities.

It should be noted that some enzymatic fractions appear in areas where there are no proteins stained with amido-schwarz. This is especially true with the fourth fraction of the MDH. This represents about two thirds of the total activity of the MDH, and its m_r is 0.401. At that level no protein fraction is found in the CSF. On the other hand, in the serum one finds at this level the β -2 globulin. The fraction which immediately follows the β -1 globulin in the CSF has a m_r of 0.348. Measuring the enzymatic activity by Wieme's method in this way enables one to show that on the 0.401 level, there exists a protein fraction which cannot be demonstrated by amido-schwarz.

Hydrosoluble Proteins of the Central Nervous System

We have effected extractions of the proteins of the grey matter and white matter of the cerebral hemispheres. The curves are very easily obtained, all the more a MDH and LDH enzymatic activity is very pronounced in brain tissue (100 times higher than in serum). After electrophoresis these extracts

TABLE 5
RELATIVE CONCENTRATIONS* OF THE DIFFERENT LACTIC AND MALIC ACID
DEHYDROGENASES IN BRAIN EXTRACTS IN 15 SAMPLES

LDH	I	II	III	IV	V	VI
LDH						
Grey matter	20.8	24.1	26.6	20.1	8.6	
White matter	21.3	27.6	25.7	19.4	7.0	
MDH						
Grey matter	12.1	12.4	12.5	53.1	7.0	4.0
White matter	14.9	12.1	12.0	50.5	8.9	3.2

* Expressed in per cent.

do not permit demonstrating the proteins on agar gel. To obtain pherograms of the cerebral proteins, more concentrated solutions have to be used. In consequence we study the enzymatic activity of the extracts of cerebral proteins on pherograms that cannot be stained by amido-schwarz. The curves thus obtained are nevertheless very well defined, and they resemble the curves obtained for serum and CSF. The relative concentrations (TABLE 5) of the different fractions are comparable to what one can observe in the serum, and we do not find, as in the CSF, a predominance of the first fraction of the LDH. The m_r (TABLE 6) for the different enzymatic fractions of the LDH and the MDH are identical with those found in the serum and in the CSF. As in the case of the serum and of the CSF, the enzymatic activities may indicate here the presence of proteins, where the amido-schwarz does not give any indication.

SHEEP

The same techniques can be used for the study of the proteins of the serum, CSF, and cerebral extracts in sheep. The results (TABLE 6) allow the following comments: (1) the general appearance of the curves for LDH and for

MDH is similar to what one observes in man (FIGURE 3); and (2) the m_r values are considerably different but show similar characteristics as in man: (a) some of the fractions of enzymatic activity appear in areas where there are no proteins stained with amido-schwarz; (b) some of the m_r are identical for LDH

TABLE 6
RELATIVE MOBILITIES OF THE DIFFERENT LACTIC AND MALIC ACID
DEHYDROGENASE ACTIVITY PEAKS IN BRAIN EXTRACTS

	Number	I	II	III	IV	V	VI
LDH							
Grey matter	15	0.881	0.665	0.468	0.239	0.044	
White matter	15	0.890	0.672	0.461	0.233	0.044	
CSF	74	0.910	0.677	0.456	0.241	0.073	
Serum	11	0.932	0.691	0.454	0.218	0.021	
MDH							
Grey matter	15	1.073	0.908	0.666	0.392	0.227	0.049
White matter	15	1.028	0.895	0.663	0.404	0.255	0.092
CSF	40	1.067	0.905	0.681	0.404	0.255	0.029

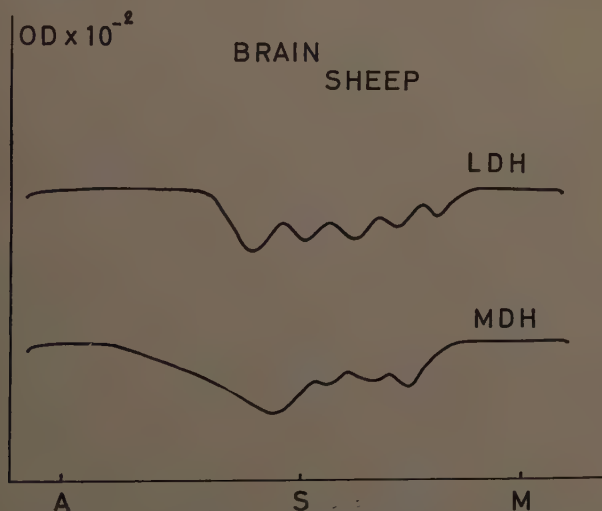


FIGURE 3. Lactic and malic dehydrogenase enzymatic activity curves in sheep brain extracts.

and MDH; and (c) the m_r of the different fractions are extremely constant in the various media which have been studied.

When comparing the m_r of the different enzymatic fractions in biological material originating from sheep to the enzymatic activities in biological material originating from man, one finds that the places occupied in the pherogram are different. The m_r of the albumin in the two species are rather near one another, but the m_r of the enzymatic activities are very different. Gen-

erally the enzymatic fractions in man are identified in an area nearer to the albumin than in sheep.

Discussion

As a rule, our results have been obtained from material originating from normal subjects. A few pathological cases have been studied. The CSF and the cerebral extracts until now hardly show any modifications in the pathological cases which we have been able to follow.

The comparison between normal and pathological material has been disappointing, but we have nevertheless been able to come to some conclusions:

(1) In the different media under study, the enzymatic activities that have been followed are all heterogeneous and are so in a similar way. The curve of enzyme electrophoresis of the LDH or of the MDH stays the same, whether we study serum, CSF or cerebral extracts. The heterogeneity of these different fractions seem to be a general rule of which we do not yet know the significance.

(2) Although we are convinced that enzymatic activity is always linked to a protein, we must recognize that in certain cases enzymatic activity is demonstrated in the pherogram at levels where we do not find protein fractions stained with amido-schwarz. This means that enzymatic activity can be a more sensitive reaction than the amido-schwarz staining for the presence of proteins.

(3) Certain protein fractions may present several enzymatic activities and especially the α -2 globulin fraction may at the same time present the activities of LDH and MDH.

(4) The relative concentrations of the various fractions can differ according to the media under study. In the CSF the first LDH fraction is the most important; in the serum, the second. This might suggest that the first fraction must also be the most important in the cerebral proteins. We have not been able to confirm this hypothesis and, in fact, our enzyme electropherograms of cerebral extracts for the white matter, as well as for the grey matter, are similar to the enzyme electropherograms of the serum.

(5) Measuring the m_r seems to us to be of the highest importance in enzyme electrophoresis. It makes it possible to show how constant the electrophoretic migration is in the different enzymes which we have studied. It seems to resist not only the conservation and the aging of the enzymes, but also most of the pathological manifestations in the neurological material that we have been able to study. On the other hand, between one species and the other, more particularly when comparing human material with material originating from sheep, we find extremely important modifications of the m_r . There is also a fact to be stressed: the m_r vary from one species to the other, but the order of succession of the fractions stays the same. Therefore, the m_r can be an important indicator for the identification of the different fractions.

Conclusions

The research we have been able to do in the serum, the CSF, and the cerebral extracts on hydrosoluble proteins originating from human species as well

as from sheep has enabled us to confirm the heterogeneousness of the dehydrogenases of the lactic and malic acid. It has also made it possible to show the importance of measuring the mobility rate of each one of these fractions for purposes of identification. We believe the method that has been used should be applied to other enzymatic activities and to other biological material. We believe that this method could yield interesting information and that, more especially, the measuring of the mobility rate provides a useful index for the demonstration of the existence of these different fractions.

References

- LOWENTHAL, A., M. VAN SANDE & D. KARCHER. 1960. The differential diagnosis of neurological diseases by fractionating electrophoretically the CSF γ -globulins. *J. Neurochem.* **6**: 51-56.
- WIEME, R. J. 1959. Studies on agar gel electrophoresis. Arscia Uitgaven N.V. Brussels, Belgium.

SEROLOGICAL DIFFERENTIATION OF AMYLASE ISOZYMES*

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From the work of previous investigators with such enzymes as lactic dehydrogenase,^{1,2,3} alkaline phosphatase,^{4,5} and phosphorylase,⁶ it is evident that one may differentiate certain isozymes (multiple molecular forms of an enzyme) by the use of antisera to one or more of the isozymes. If one of the isozymes is inhibited or precipitated by a given antiserum and another is not inhibited or precipitated (or is inhibited to a greater or lesser degree), then one may say unequivocally that the two isozymes are structurally, as well as immunologically, different. On the other hand, if two isozymes are inhibited or precipitated equally by a given antiserum, one can say only that they *may* be identical, although in such a situation the isozymes are certainly similar. Using the Ouchterlony technique, Li showed that human and monkey growth hormones are immunologically identical,⁷ although his earlier chemical studies⁸ on the two hormones showed that they differed somewhat in structure.

Formation of antibodies to malt β -amylase⁹ and microbial amylases¹⁰ had been reported. We were interested in relationships among mammalian amylases, however, and used hog pancreatic amylase (HPA) and human salivary amylase (HSA) as antigens. Antisera to HPA were formed in the rabbit,¹¹ rooster,¹² and the rat, and antisera to HSA in the rabbit. By use of these antisera we have studied the relationships of the amylases of the different organs of a given species and also the relationships of amylases of a given organ of different species.

Experimental

Production of antibodies. The antigens used¹³ were crystalline hog pancreatic amylase† and purified human salivary amylase prepared from pooled fresh human saliva by the method of Meyer *et al.*¹⁴ The animals to be injected were first bled weekly to obtain normal sera, which was preserved by freezing at -18° C. The amylase, in aqueous solution or suspension was emulsified with Freund's adjuvants,¹⁵ light mineral oil and Arlacel A, omitting the heat-killed bacteria in most of the cases. For rabbits and roosters, the initial injections in adjuvants contained 20 to 30 mg. of amylase given intradermally, and successive injections at 10 to 14 day intervals contained 10 mg. of amylase in aqueous solution. For rats, the initial injection was 10 mg. and successive doses, 5 mg. each. Blood samples were obtained from each animal before each injection, and the amylase-inhibiting capacity of each antiserum was determined. Maximum amylase-inhibiting antibody levels were usually obtained after three or four injections.

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† Obtained from the Worthington Biochemical Corporation, New York, N.Y.

Demonstration of amylase antibodies. Equal volumes of the amylase-containing solutions and diluted antisera (1:20 or 1:100 dilutions in 0.9 per cent NaCl) were mixed and allowed to stand for 40 min. to insure maximal inhibition of the amylase. The amylase-containing solutions were dilutions of the original antigen, sera, urine, saliva, or homogenized tissues with 0.9 per cent NaCl, adjusted so that they contained the same amount of amylase (0.1 to 0.2 amylase units per ml.). One ml. aliquots of the mixtures were then used to determine the amylase activities. In each case, control experiments were run using diluted normal rabbit serum obtained from the same rabbit whose antiserum was being tested, and the activity of the amylase being tested was determined in this mixture. With the activities of the amylase-containing solutions determined in the presence of normal rabbit serum and the endogenous amylase activity of the rabbit antiserum, each determined separately, it was possible to calculate the inhibition of the amylase being tested.

The presence of amylase antibodies in rabbit antisera to both HPA and HSA was demonstrated by using Oudin's method¹⁶ of layering a solution of the antigen on top of agar-antiserum gels.

Analytical methods. Amylase analyses of HPA and HSA, the normal and immune sera, and the other tissues and body fluids used were carried out as previously described¹⁷ with Van Loon's amylolytic method.¹⁸

Sources of amylases. The sources of HPA and HSA have already been described. Extracts containing amylases were prepared from the appropriate organs by homogenizing with cold (5°) 0.9 per cent NaCl as previously described.¹⁷ Serum, urine, and other fluids used were diluted appropriately with 0.9 per cent NaCl.

Results

We have been able to produce amylase-inhibiting antibodies to HPA in the rabbit, rooster, and rat and to HSA in the rabbit. Attempts to produce antibodies to HSA in the rat were unsuccessful. In all cases, only a few injections were given in order to avoid the possibility that the antisera might become less specific with additional injections.¹⁹ Also with the rooster and the rat, it was found that after several injections, the amylase-inhibiting capacity of the antisera began to decrease. In these cases, those antisera with maximum titers were used in succeeding experiments. The levels obtained in each case are found in the various tables. With normal sera from the various species, no inhibition of either of the antigens or other amylases tested was noted.

The rate of reaction of antigen with antibody was apparently not as rapid as indicated in some cases.⁶ After we had experienced some unexplained variations in preliminary trials, investigation (TABLE 1) showed that at least 30 to 40 min. at room temperature were necessary for complete reaction before adding the antigen-antibody mixture to the starch substrate.

In all animals whose amylases were affected by the various antisera, it was seen that whereas the pancreatic and salivary amylases of a given species were inhibited quite strongly by the appropriate antisera, the liver amylase was not inhibited significantly (TABLES 2, 3, 4, and 5). This includes the several

cases where certain amylases were inhibited by heterologous antisera. Control experiments with ternary systems of pancreatic amylase, liver amylase, antiserum and serum amylase, liver amylase, and antiserum showed that the pancreatic and serum amylases were inhibited equally as much in the presence as in the absence of liver extract. Thus the lack of inhibition of liver amylase was not due to interference by other liver proteins. Serum amylases were

TABLE 1*
CHANGE IN INHIBITION OF HOG PANCREATIC AMYLASE
WITH TIME OF REACTION WITH ANTISERUM

Time (min.)	Inhibition Dilution of antiserum	
	1:20 (%)	1:100 (%)
0	25	9
20	87	58
40	96	78
60	96	81
80	94	86
100	94	85

* Reproduced by permission of the *Journal of Biological Chemistry*.¹¹

TABLE 2*
INHIBITION OF AMYLASES BY RABBIT ANTISERA TO HOG PANCREATIC AMYLASE

Amylase from	Hog		Dog		Rat	
	Dilution of antiserum					
	1:20 (%)	1:100 (%)	1:20 (%)	1:100 (%)	1:20 (%)	1:100 (%)
Pancreas	95	91	38	8	9	5
Saliva	95	—	—	—	5	2
Serum	93	75	35	13	4	3
Liver	4	1	13	8	3	5
Kidney	70	35	22	15	5	6
Spleen	93	70	66	39	24	15

* Reproduced by permission of the *Journal of Biological Chemistry*.¹¹

TABLE 3*
INHIBITION OF AMYLASES BY ROOSTER ANTISERA TO HOG PANCREATIC AMYLASE

Amylase from	Per cent inhibition at 1:20 dilution of antisera		
	Hog	Dog	Rat
Pancreas	72	8	48
Serum	39	18	0
Liver	6	16	5

* Reproduced by permission of the *Biochimica et Biophysica Acta*.¹²

significantly inhibited by homologous antisera, but in all cases this inhibition was less than that of the corresponding pancreatic or salivary amylase. However (TABLE 6), when sera were obtained from human subjects with pancreatitis, the amylase of these sera was inhibited 73 per cent, substantially the same inhibition found for human pancreatic and salivary amylases.

TABLE 4
INHIBITION OF AMYLASES BY RAT ANTISERA TO HOG PANCREATIC AMYLASE

Amylase from	Per cent inhibition at 1:100 dilution of antisera				
	Hog	Man	Monkey	Dog	Rabbit
Pancreas	68	2	1	3	29
Saliva	60	0	7	—	—
Serum	50	13	8	2	12
Liver	2	7	0	0	3
Urine	—	0	—	—	—

TABLE 5
INHIBITION OF AMYLASES BY RABBIT ANTISERA TO HUMAN SALIVARY AMYLASE

Amylase from	Per cent inhibition at 1:20 dilution of antisera				
	Man	Monkey	Chimpanzee	Hog	Rat
Pancreas	75	75	—	1	63
Saliva	78	77	75	—	16
Serum	45	60	—	0	0
Urine	50	—	—	—	—
Liver	7	0	—	3	0
Fallopian tube cyst	82	—	—	—	—

TABLE 6
COMPARISON OF AMYLASES IN NORMAL AND PANCREATITIS HUMAN SERA

Amylase from human	Per cent inhibition at 1:20 dilution of antisera
Pancreas	75
Saliva	78
Normal serum	45
Pancreatitis serum	73

In man, the inhibitions of the serum and urinary amylases were essentially equal. These data support the assumption that the amylase found in the urine had filtered through the glomeruli from the plasma.²⁰

We had expected that monkey (Rhesus) and chimpanzee amylases would be inhibited by the antisera to HSA but to a lesser degree than the corresponding human enzymes. Thus it was surprising to find that human, chimpanzee, and monkey salivary amylases and human and monkey pancreatic amylases were inhibited equally strongly (TABLE 5).

It was also surprising to find such marked inhibition of rat pancreatic amylase by the antisera to HSA and yet only slight inhibition of rat salivary amylase. This case was an exception to the general finding that the pancreatic and salivary amylases of a given species were inhibited equally.

The presence of antibodies in antisera to both HPA and HSA was demonstrated by precipitin reactions using Oudin's method although the reaction could not be demonstrated in liquid media. In the various dilutions of antisera and antigens,¹¹ only a single band of precipitate was seen where a reaction was noted. In all controls with antigens and normal sera, no precipitate was seen at any of the dilutions used.

Discussion

We have demonstrated that hog pancreatic and human salivary amylases are antigenic and that the antibodies formed have amylase-inhibiting properties. These findings are in contrast to those with antibodies to dog phosphatases⁴ where the antigen-antibody complexes were enzymatically active. This indicates that the enzymatic site in the amylases is part of or located close to the antigenic site on the amylases and has been blocked or covered by the large antibody molecules.

In most cases, the pancreatic and salivary amylases of a given species were immunologically identical and therefore possibly also structurally identical. The liver amylases in all cases however, were immunologically, and therefore structurally, different from their corresponding pancreatic and salivary amylases. It was also evident that the amylase found in the normal serum of any species is derived at least partially from some source other than the pancreas or salivary glands, a conclusion previously reached for rats from data on pancreatectomized-salivarectomized rats.¹⁷

It has been assumed for years that the increase in serum amylase during pancreatitis is due to the liberation of pancreatic amylase into the blood. The data in TABLE 6 seem to substantiate this assumption since the added amylase is immunologically similar to pancreatic amylase.

One of the advantages of the immunological technique in differentiating amylase isozymes is that one can obtain useful information without having to purify all the amylases used. Since the reaction between antigen and antibody is specific and is not usually affected by extraneous protein, one can test the antisera obtained on unpurified preparations of the enzymes. It has been demonstrated that the inhibition of lactic dehydrogenases from rabbit organs by antirabbit muscle lactic dehydrogenase^{2,3} was essentially the same for unpurified organ extracts as for the corresponding purified enzymes. We have obtained similar results with both HPA and HSA.

Acknowledgments

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References

1. MANSOUR, T. G., E. BUEDING & A. B. STAVITSKY. 1954. The effect of a specific anti-serum on the activities of lactic dehydrogenase of mammalian muscle and of *Schistosoma mansoni*. Brit. J. Pharmacol. **9**: 182.
2. NISSELBAUM, J. & O. BODANSKY. 1959. Reactions of lactic dehydrogenase from various rabbit organs with antirabbit muscle lactic dehydrogenase. J. Biol. Chem. **234**: 3276.
3. PLAGEMANN, P. G. W., K. F. GREGORY & F. WRÓBLEWSKI. 1960. The electrophoretically distinct forms of mammalian lactic dehydrogenase. I. Distribution of lactic dehydrogenases in rabbit and human tissues. J. Biol. Chem. **235**: 2282.
4. SCHLAMOWITZ, M. 1954. Specificity of dog intestinal phosphatase antiserum. J. Biol. Chem. **206**: 361.
5. SCHLAMOWITZ, M. & O. BODANSKY. 1959. Tissue sources of human serum alkaline phosphatase, as determined by immunochemical procedures. J. Biol. Chem. **234**: 1433.
6. HENION, W. F. & E. W. SUTHERLAND. 1957. Immunological differences of phosphorylases. J. Biol. Chem. **224**: 477.
7. LI, C. H., N. R. MOUDGAL & H. PAKOFF. 1960. Immunochemical investigations of human pituitary growth hormone. J. Biol. Chem. **235**: 1038.
8. LI, C. H. & H. PAKOFF. 1956. Preparation and properties of growth hormone from human and monkey pituitary glands. Science. **124**: 1293.
9. LUERS, H. & F. ALBRECHT. 1924. Anti-amylase. Fermentforschung. **8**: 52.
10. WADA, T. 1959. An immunochemical study of microbial amylase. II. J. Biochem. (Tokyo). **46**: 329.
11. McGEACHIN, R. L. & J. M. REYNOLDS. 1959. Differences in mammalian amylases demonstrated by enzyme inhibition with specific antisera. J. Biol. Chem. **234**: 1456.
12. McGEACHIN, R. L. & J. M. REYNOLDS. 1960. Inhibition of amylases by rooster antisera to hog pancreatic amylase. Biochim. et Biophys. Acta. **39**: 531.
13. CALDWELL, M. L., M. ADAMS, J. T. KUNG & G. C. TORALBALLA. 1952. Crystalline pancreatic amylase. II. Improved method for its preparation from hog pancreas glands and additional studies of its properties. J. Am. Chem. Soc. **74**: 4033.
14. MEYER, K. H., E. H. FISCHER, A. STAUB & P. BERNFELD. 1948. Sur les enzymes amylolytiques X. Isoelement et cristallisation de l' α -amylase de salive humaine. Helv. Chim. Acta. **31**: 2158.
15. COHN, M. 1952. Production of antibodies in experimental animals. In Methods in Medical Research. **5**: 275. Year Book Publishers. Chicago, Ill.
16. OUDIN, J. 1952. Specific precipitation in gels and its application to immunochemical analysis. In Methods in Medical Research. **5**: 340. Year Book Publishers. Chicago, Ill.
17. McGEACHIN, R. L., J. R. GLEASON & M. R. ADAMS. 1958. Amylase distribution in extrapancreatic, extrasalivary tissues. Arch. Biochem. Biophys. **75**: 403.
18. VAN LOON, E. J., M. R. LIKINS & A. J. SEGER. 1952. Photometric method for blood amylase by use of starch-iodine color. Am. J. Clin. Pathol. **22**: 1134.
19. HOOKER, S. B. & W. C. BOYD. 1934. The existence of antigenic determinants of diverse specificity in a single protein. II. In two natural proteins: crystalline duck egg albumin and crystalline hen egg albumin. J. Immunol. **26**: 469.
20. McGEACHIN, R. L. & L. A. HARGAN. 1956. Renal clearance of amylase in man. J. Appl. Physiol. **9**: 129.

Discussion of the Paper

A. SAMUELS (*Department of Pathology, Dartmouth Medical School, Hanover, N.H.*): McGeachin seems to suggest that there may be immunologic identity between the organ amylases of different species, based on his evidence that the pancreatic amylases from different species were inhibited to the same extent (almost completely) by an excess of antiserum to one of the species. I feel that this procedure would mask the differences between cross-reacting proteins, and that the more sensitive method of titrating the enzymes with the antisera and if possible determining a binding constant would be a more acceptable process of determining similarities or differences.

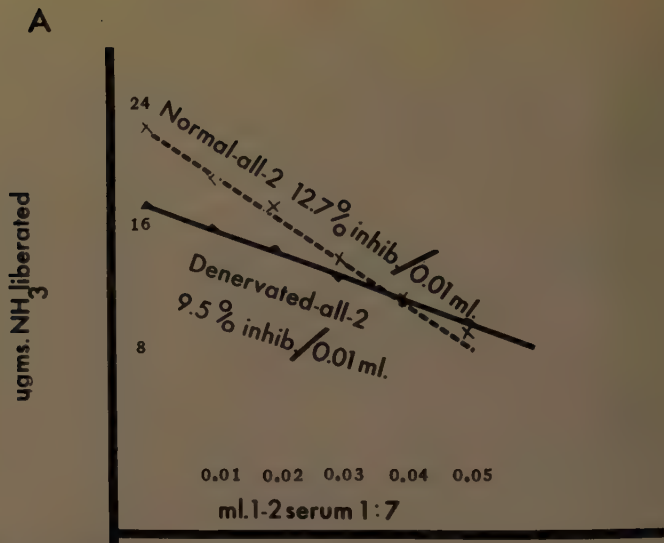


FIGURE 1a. Differential inhibition of normal and denervated gastrocnemial-muscle adenylic-acid deaminase by diluted antiserum. Constant amounts of enzyme and increasing amounts of antisera were incubated at 37°C . for 1 to 3 hours in 0.3 ml. of 0.5 MKl or KCl-PO_4 buffer ($\text{T}/2 = 0.54$, pH 6.3). An amount of 0.8 ml. KCl-succinate buffer (pH 5.9) containing 1.5 mg. adenylic acid was then added, and the reaction stopped with TCA at the end of 5 min. The NH_3 liberated was determined by nesslerization.

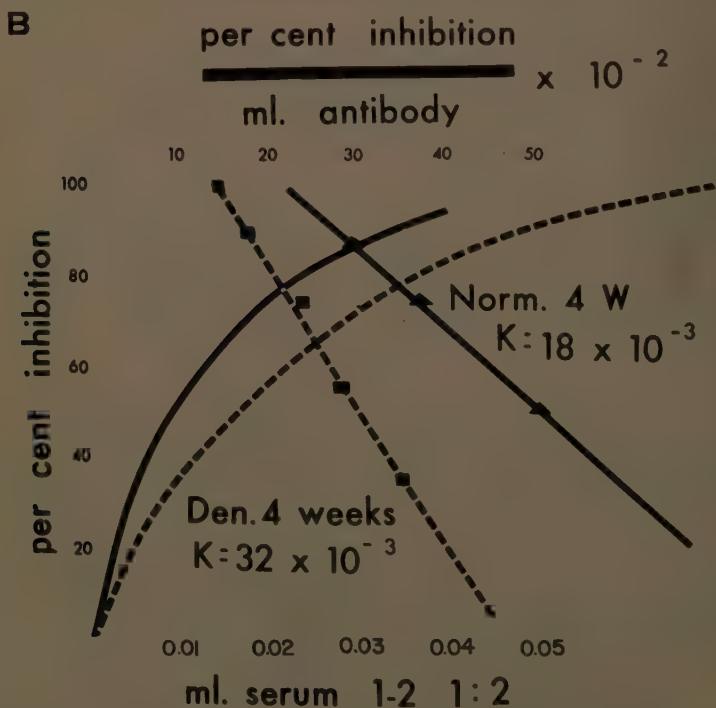


FIGURE 1b. The data are the same as in FIGURE 1a but with more concentrated antiserum (see text).

As an example of what is meant by the titration of the enzyme by cross-reacting antisera and as a presentation of another molecular form that enzymes may take, I present figures that depict the inhibition of 5'-adenylic acid deaminase from normal skeletal muscle of the chicken and a preparation derived from denervated muscle (contralateral) by antibody made to deaminase from normal chicken skeletal muscle in rabbits. FIGURE 1*a* shows the linear course of inhibition obtained with diluted antisera when the enzyme is inhibited only 50 per cent by the antibody. The enzyme of the normal muscle is inhibited to a greater extent by the antibody than the enzyme prepared from denervated muscle. The inhibition of the enzyme is linear up to 50 per cent inhibition, then curves sharply as 100 per cent inhibition is approached.* The curves in FIGURE 1*b* (here plotted as per cent inhibition) show the data obtained when the antiserum is used in more concentrated form. Straight lines may be obtained if, instead of plotting the per cent inhibition versus milliliter of antiserum, the per cent inhibition (bound enzyme) versus per cent inhibition/milliliter antiserum is plotted. Algebraic manipulation shows that the slopes of these lines are inversely proportional to the binding constant between the enzyme and antibody. Here again it is seen that the enzyme from a chicken gastrocnemial muscle, denervated for four weeks has much less affinity for the antibody made to normal muscle adenylic acid deaminase.

* SAMUELS, A. 1961. Arch. Biochem. Biophys. **92**: 497.

IMMUNOCHEMICAL ANALYSIS OF THE MULTIPLE FORMS OF BOVINE RIBONUCLEASE

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Introduction

The realization that crystallized bovine pancreatic ribonuclease was not a single protein was perhaps the final blow to the belief that crystallization of a protein constituted a criterion of homogeneity. The crystallized enzyme was shown to be resolvable into several components by partition and ion exchange chromatography.^{1,2a,b} This heterogeneity remained undetected in experiments based on free electrophoresis,^{3a,b} but was apparent in zone electrophoresis.⁴ As a result of this heterogeneity, enzyme activity did not reside in one fraction only, but was clearly attributable to two fractions, designated ribonuclease A and B. A third and minor component with ribonuclease activity was also detected. More recently, it has been found that ribonuclease B can be separated by chromatography on carboxymethyl cellulose into two components and that crystallized ribonuclease contains, in addition to the proteins, a component which is not a protein and which may be a nucleotide.⁵ Thus, three to four protein fractions⁶ with ribonuclease activity can be separated by chromatography, at present. The principal component is ribonuclease A; a second component, ribonuclease B, may contribute 10 to 50 per cent of the total protein but may itself consist of two components. One or two other components of ribonuclease are present in small quantities.

That this multiplicity of ribonucleases extracted from the bovine pancreas is not a unique property of the bovine enzyme, became evident by the isolation of eight ribonucleases from the pancreas of the sheep.⁶

As soon as the heterogeneity of crystallized ribonuclease was observed, the possibility was considered that it was a result of the purification procedures which involved exposure to 0.25 *M* H₂SO₄ and heating to 100° C.,⁷ and it was demonstrated that the principal components of ribonuclease are, in all probability, already present in the original pancreatic material.^{1,2a,2b}

The chemical differences between the different ribonucleases are not yet established; it has been proposed that ribonuclease A and B differ in one of their carboxylic groups and that this is the only difference in their respective amino acid composition,⁸ some differences in enzyme action have also been claimed.⁹ Some of the ribonucleases separated on carboxymethyl cellulose may be identical in primary structure and may differ only in their secondary and tertiary structures.¹⁰

Six Components of Ribonuclease Demonstrable by Immuno-electrophoresis

The immunochemical analysis of crystallized ribonuclease, carried out by double diffusion in agar, reveals the presence of one and the same antigen in

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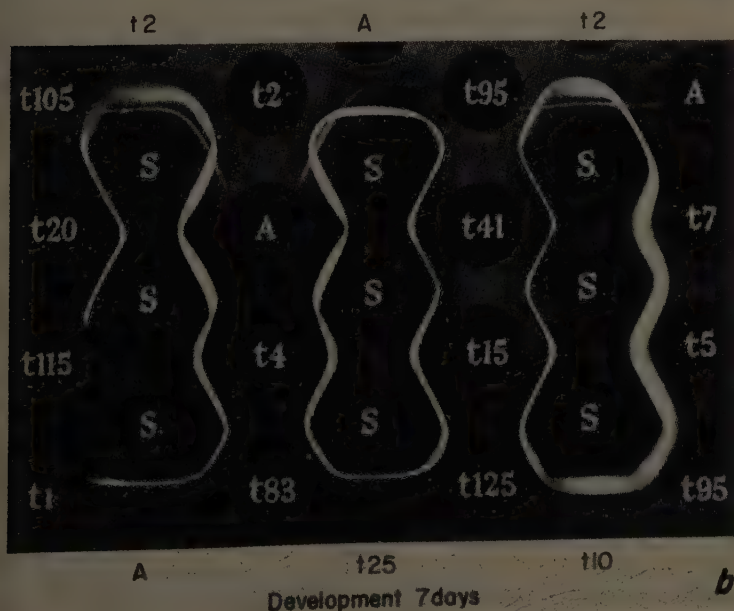
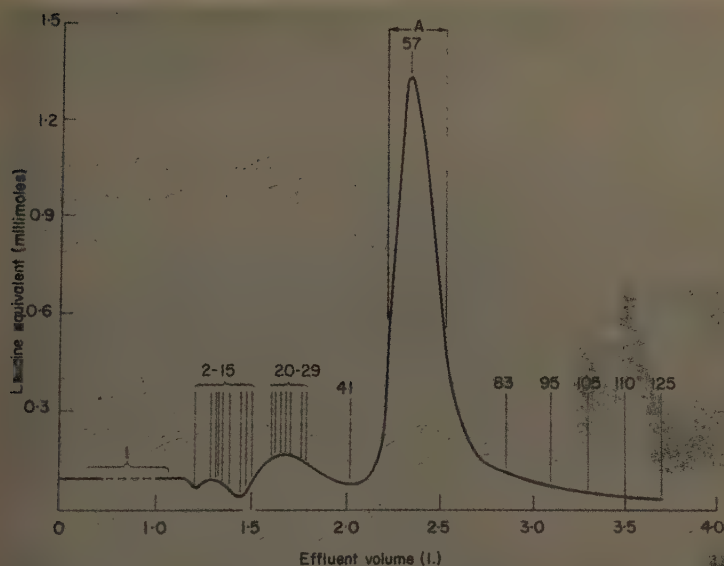


FIGURE 1. Demonstration of an antigen unrelated to ribonuclease in bovine pancreatic ribonuclease. (a) Chromatography² of bovine pancreatic ribonuclease (Armour 381-059) on Amberlite IRC-50 (XE 64). (b) Interaction in agar after double diffusion of chromatographic fractions of ribonuclease (a) and ribonuclease antiserum showing the presence of an antigen unrelated to ribonuclease in the first fraction eluted from the column of Amberlite IRC-50. Key: t = tube number.

all the fractions of ribonuclease as well as the presence of a second "independent" antigen in the first fraction of ribonuclease eluted by chromatography on Amberlite columns at pH 6.45^{11,12} (FIGURE 1). Electrophoresis in agar gels of the crystallized material, followed by diffusion of antibody from channels parallel to the direction of electrophoretic migration,^{12,14} showed a continuous zone with two maxima. The "independent" antigen appeared as a component with a mobility close to that of the slower of the two antigenically identical components¹¹ (FIGURE 2).

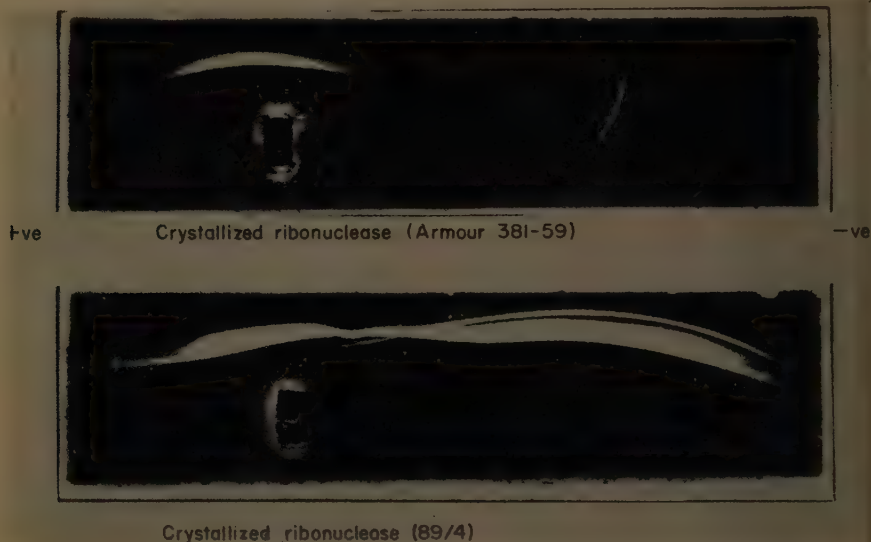


FIGURE 2. Electrophoresis in agar of bovine pancreatic ribonuclease (Armour 381-059 and ribonuclease 89/4) showing (for ribonuclease 89/4) the presence of an antigen unrelated to ribonuclease and the migration of ribonuclease with two different mobilities. The median channel, containing the antiserum, was at the top of each of these two photographs. The direction of electrophoresis of all the components is towards the anode; endosmosis in the opposite direction retards or reverses the actual displacement of components. The component nearest the anode has the highest mobility; the component nearest the cathode has the lowest mobility.

The "independent" antigen can be separated by stepwise elution of crystallized ribonuclease from carboxymethyl cellulose (0.005 *M* Tris buffer, pH 8.0) and can thus be prepared virtually free of ribonuclease. In addition, a second component of the "independent" antigen, indistinguishable from the first by double diffusion or by electrophoresis in agar, appears on elution with the same buffer containing 0.054 *M* NaCl, and may be partly responsible for the complexity of the ribonuclease B revealed by this technique (FIGURE 3; see also FIGURE 11).

The two enzyme components, migrating with different mobilities, but being apparently antigenically identical, can be correlated with components identified by chromatographic elution. It is difficult to prepare ribonuclease B

free from ribonuclease A. Fortunately, a preparation of ribonuclease (Armour 381-062) was available which contained a large proportion of ribonuclease B and from which preparations of ribonucleases A and B could be separated which did not contain detectable amounts of ribonuclease B or A respectively. Immuno-electrophoresis of these two preparations showed that ribonuclease A was the faster migrating component and that ribonuclease B migrated more

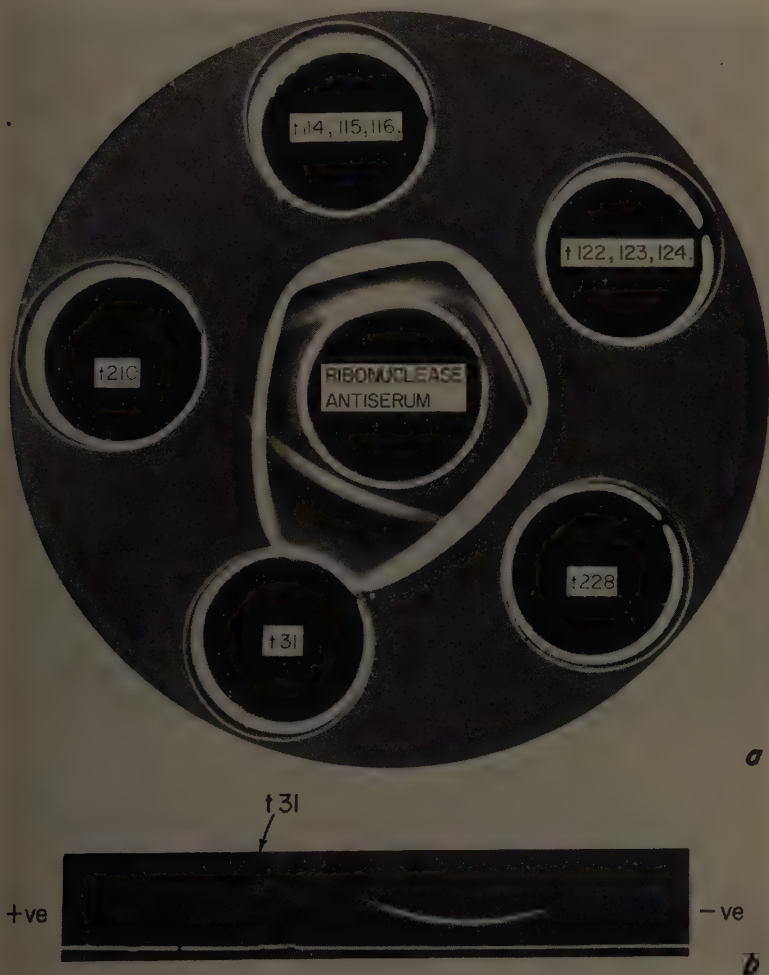


FIGURE 3. "Independent" antigen purified by chromatography on carboxymethyl cellulose (stepwise elution). Fractionation of ribonuclease is shown in FIGURE 11 (1b) where the tube numbers indicated in this figure are marked on the chromatogram. Two chromatographic components of the independent antigen are demonstrated by double diffusion. One of these components is eluted in a high state of purity (t 31), the other is eluted together with ribonuclease B (t 114-116, t 122-124). (a) Double diffusion (diffusion of antigens, t 31, t 114-116, t 122-124, t 210, t 228) against ribonuclease antibody. (b) Electrophoresis in agar (t 31) followed by diffusion of antibody from channels parallel to the direction of the current.

slowly (FIGURE 4). While in this experiment ribonuclease A migrated as a single component, ribonuclease B (FIGURES 3 and 4) showed some heterogeneity in that it seemed to contain a component antigenically identical with ribonuclease B, which had a mobility intermediate between A and B. In another

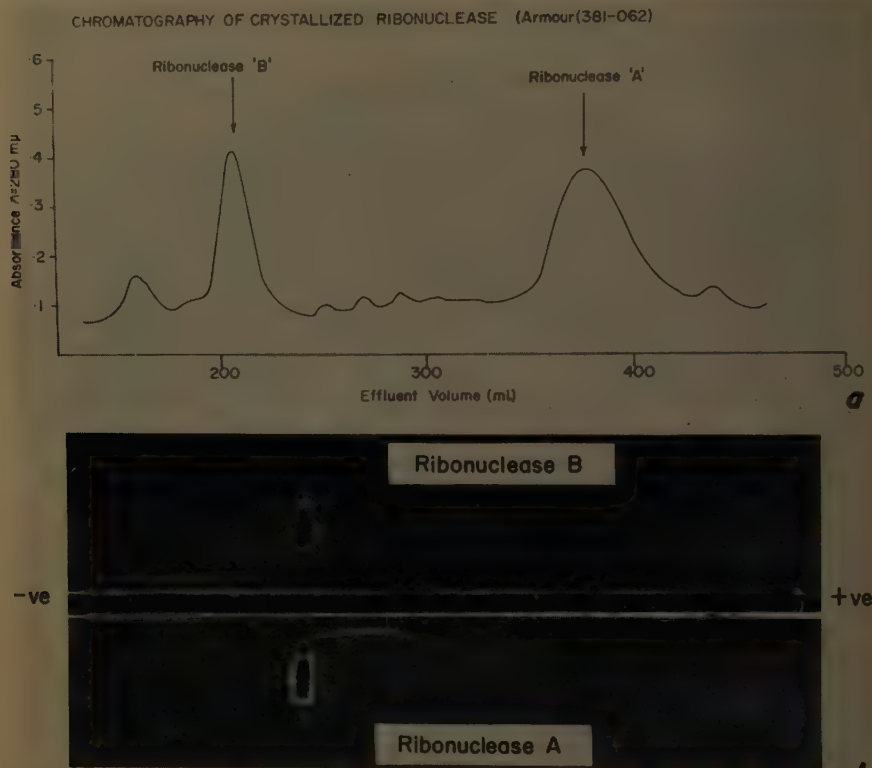


FIGURE 4. The immunoelectrophoresis of ribonucleases A and B. The direction of electrophoresis of all the components is towards the anode; endosmosis in the opposite direction retards or reverses the actual displacement of components. The component nearest the anode has the highest mobility; the component nearest the cathode has the lowest mobility. (a) Ribonucleases A and B were prepared from crystallized ribonuclease (Armour 381-062) by chromatography on Amberlite IRC-50 (XE 64). (b) Ribonuclease A (lower part of photograph) and B (upper part of photograph) were subjected to electrophoresis in agar (glycine buffer pH 8.4),¹⁴ subsequently, ribonuclease antiserum was allowed to diffuse into the agar from channels parallel to the direction of electrophoresis.

preparation of ribonuclease A, a molecular species was found that appeared to be immunologically identical with ribonuclease A but which migrated with a mobility lower than that of ribonuclease A (FIGURE 5). This molecule had a somewhat greater mobility than the faster component of ribonuclease B already described (FIGURE 4). It was eluted from Amberlite resin after B and before the peak of A had been eluted, as may be seen by immunoelectrophoresis of sam-

Electrophoresis in agar followed by diffusion of ribonuclease antiserum from channels parallel to the direction of migration

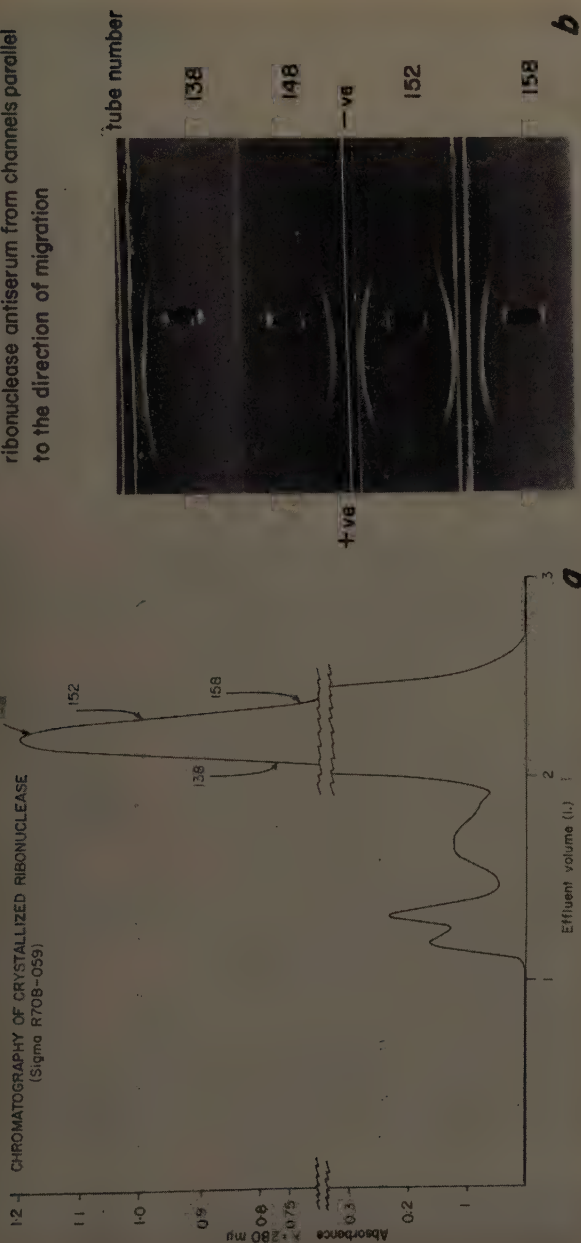


FIGURE 5. An electrophoretic component of ribonuclease intermediate in mobility between ribonucleases A and B. The direction of electrophoresis of all the components is towards the anode; endosmosis in the opposite direction slows down or reverses the actual displacement of components. The component nearest the anode has the highest mobility; the component nearest the cathode has the lowest mobility. (a) Chromatography of crystallized ribonuclease (Sigma R70B-059) on Amberlite CG-50 Type II by the method of Hirs *et al.*²⁶ (b) Electrophoresis in agar (glycine buffer pH 8.4)¹⁴ of fractions of ribonuclease A (tubes 138, 148, 152, 158 of chromatography experiment) followed by diffusion of ribonuclease antiserum from channels parallel to the direction of electrophoresis.

ples taken at different stages during the elution of ribonuclease A (FIGURE 5). Thus four constituents of ribonuclease can be distinguished by immunoelectrophoresis of fractions of ribonuclease; only the two principal components could be demonstrated in all unfractionated preparations. A fifth component was observed in several unfractionated preparations of crystallized ribonuclease, but could be demonstrated only if relatively high concentrations of crystallized ribonuclease were subjected to electrophoresis. This component, shown in FIGURE 6, migrated with a mobility greater than ribonuclease A.

We had thus observed a total of five components of ribonuclease which, by double diffusion in agar, appeared to be identical but which showed different mobilities on electrophoresis in agar at pH 8.4.¹⁴ Furthermore, the presence of an antigenic component has already been mentioned which is not a ribonuclease.

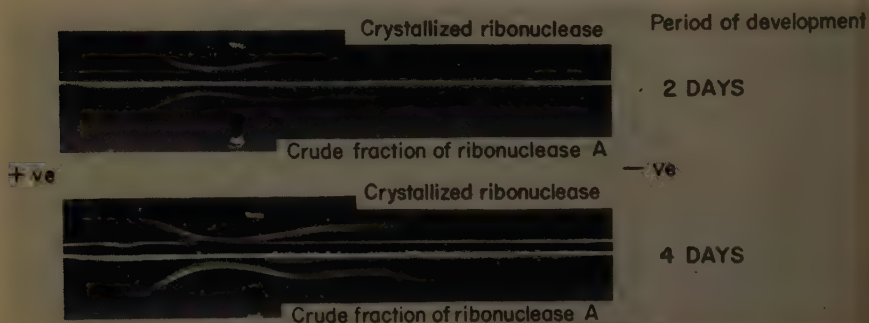


FIGURE 6. A component of ribonuclease with mobility greater than ribonuclease A. Crystallized ribonuclease (Sigma R50B-095) and a crude fraction of ribonuclease A were subjected to electrophoresis in agar (glycine buffer pH 8.4).¹⁴ Subsequently ribonuclease antiserum was allowed to diffuse into the agar from channels parallel to the direction of electrophoresis. After 2 days of development ribonuclease A and B had formed zones of precipitation. A zone of precipitation appeared after 3 to 4 days indicating the presence of a fast component in the crystallized preparation. This component was not present in the crude fraction of ribonuclease A.

The complexity of the enzyme preparations was, therefore, shown to be somewhat greater than that revealed by chromatography.

In addition to the components thus far described, some other components can be observed in preparations freeze-dried and stored at room temperature for long periods. The existence of these components is doubtlessly the outcome of denaturation. In immunoelectrophoresis their presence is revealed by the appearance of diffuse asymmetrical zones in place of the sharp distinct zones. The maxima of these diffuse zones cannot be accurately localized. The chromatography on Amberlite of such preparations (a preparation of denatured ribonuclease A and of a denatured preparation of crystallized ribonuclease) shows fractions not seen in enzyme preparations stored in the cold and in vacuo (FIGURE 7). A large proportion of the protein is eluted very quickly and has no detectable enzyme activity (I), a second fraction (II) of slightly higher activity is finally followed by a fraction (III), which resembles ribonuclease A in its elution characteristics (FIGURE 7ii, iii) and specific activ-

ity, but which can be shown by mixing the denatured preparation with native ribonuclease A (FIGURE 7i) to be eluted from Amberlite sooner than is native ribonuclease A (FIGURE 7iv).

The multiple forms of native bovine pancreatic ribonuclease thus far described, have been distinguished by their relative electrophoretic mobility. If the ribonucleases were compared by double diffusion of antigen and antibody in agar (FIGURE 1) they seemed to be identical. Double diffusion is not a very discriminating method of distinguishing between molecules that show very slight differences. We know, from earlier model experiments that ribonuclease and azo-ribonuclease, which differ by the introduction of only 7 groups of azo-para-amino sulphonic acid, give a reaction of identity if examined by double diffusion in agar. As many as 18 groups of para-amino sulphonic acid have to be introduced into ribonuclease before a distinct reaction of partial identity can be seen. In this particular situation, the quantitative precipitin assay proved a much more sensitive method in that it did show differences between native ribonuclease and azo-ribonuclease, containing 7 azo groups.¹⁵

The Quantitative Precipitin Reaction with Ribonuclease A

In examining the reaction between ribonuclease A and ribonuclease antisera,¹⁵ it was first observed that antigen and antibody seemed to coexist in supernatants throughout the range of excess antibody. This phenomenon was not detected if the supernatant from the precipitates of antigen and antibody were examined by the usual precipitation test, but was observed when the supernatants were assayed by measurement of enzyme activity and by agglutination of tanned erythrocytes sensitized with ribonuclease A. One ribonuclease-antiserum, however, reacted with ribonuclease A in antibody excess with complete precipitation of antigen,¹¹ it gave an equivalence zone and could be used to determine the ratios between antigen and antibody in the precipitates separated from mixtures of different initial composition. The results of experiments with this serum are given in TABLES 1 and 2.

Before further quantitative aspects of the interaction of ribonuclease with antibody can be considered, some mention must be made of the difficulty of estimating the total amount of antibody in any given precipitate. Such estimates are based on the assumption that the precipitate of antigen and antibody in the zone of antibody excess contains all the antigen that was present in the original mixture. This assumption is clearly incorrect with respect to all ribonuclease antisera under examination. Nevertheless this assumption is being made in all estimates of the total antibody removed. These estimates are therefore always only approximate, but since it has been shown (see TABLES 1 and 2) that the antigen content of the precipitates in the relevant part of the zone of antibody excess is between 3 and 5 per cent, the error caused by this procedure will not be very great.

The Differences Between Ribonucleases A and B Revealed by Precipitin Test

The amount of total N precipitated from mixtures of antibody with ribonuclease A and from mixtures with ribonuclease B were compared. The two

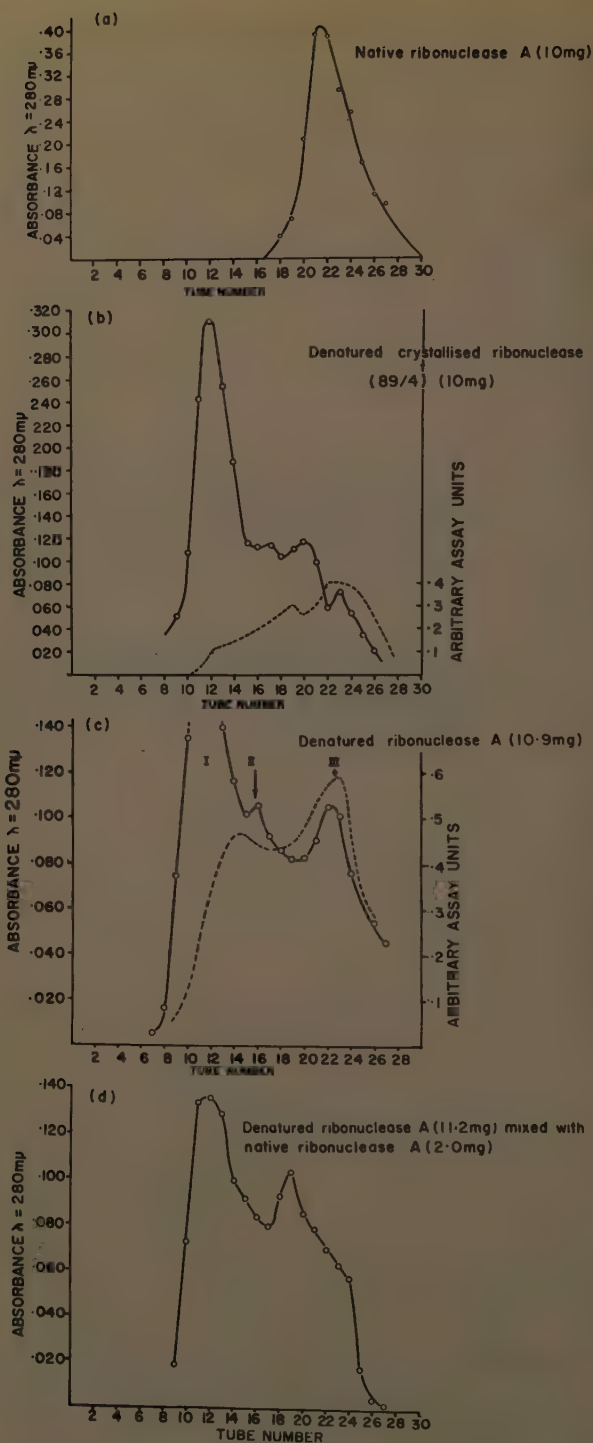


FIGURE 7.

antigens are found either to precipitate the same amount of antibody or, less frequently, the amount of antibody precipitated by ribonuclease A was about 5 to 15 per cent greater than the amount of antibody precipitated by ribonuclease B¹¹ (FIGURE 8). This difference could be very greatly increased if the two antigens were allowed to interact with antisera from which part of the antibody had been previously removed by the addition of ribonuclease A. The residual antibody gave a much larger precipitate with ribonuclease A than it did with ribonuclease B (FIGURE 9). As can be seen from FIGURE 9 the

TABLE 1
PRECIPITATION OF RIBONUCLEASE A BY RABBIT ANTIBODY
Addition of Increasing Amounts of Ribonuclease to 0.05 ml.
of Serum 619. Rn = Ribonuclease A

Antigen N	Total N precipitated	Ratio antibody N to antigen N in precipitate	Molecular ratio* (antibody to antigen) in:		Tests on supernatant			
			Precipitate	Supernatant	Antigen		Antibody	
					Flocculation	Enzyme activity as $\mu\text{g. N RnA}$ per 3 ml.	Flocculation	Agglutination titer
1.0	34.4	33.4	2.86	—	—	—	+	
2.0	66.8	32.6	2.79	—	—	—	+	200
3.05	101.0	32.1	2.74	—	—	—	+	200
3.95	118.9	29.1	2.49	—	—	—	+	200
4.9	135.2	26.4	2.26	—	—	—	+	50
5.9	148.2	24.0	2.05	—	—	—	—	50
7.4	177.6	22.9	1.96	—	—	—	—	10
8.9	185.7	19.9	1.70	—	—	—	—	1
9.8	191.4	18.5	1.58	—	—	—	—	1
10.2	188.4	17.7	1.51	—	+	0.5	—	1
13.2	138.5	15.9	1.36	0.87	+	3.8	—	1
15.3	106.4	16.7	1.43	0.75	+	7.2	—	1
17.3	83.5	17.8	1.52	0.71	+	10.2	—	1
19.3	69.2	17.0	1.45	0.65	+	13.6	—	1

* Calculated assuming identical N content for ribonuclease and antibody, a molecular weight 13,680 for ribonuclease (Hirs *et al.*²⁰) and of 160,000 for rabbit antibody.

difference in the amounts of antibody precipitated is considerable and well outside the limits of experimental error.

FIGURE 7. The chromatographic separation of a preparation of denatured ribonuclease A and a denatured preparation of crystallized ribonuclease on Amberlite IRC-50 (XE 64). Activity of ribonuclease was determined by allowing samples of the enzyme to act for 10 min. on yeast ribose nucleic acid; uranyl acetate was then added to the system, the resulting precipitate separated by centrifugation, and the absorbence of the supernatant at 260 μm was plotted in arbitrary units of activity. (a) The rechromatography of native ribonuclease A. (b) The chromatography of denatured crystallized ribonuclease. (c) The chromatography of denatured ribonuclease A. (d) The chromatography of a mixture of denatured ribonuclease A with native ribonuclease A showing the appearance of a shoulder on the last chromatographic component and hence a difference in the chromatographic behavior between the last component of denatured ribonuclease A to be eluted from Amberlite IRC-50 (XE 64) and of native ribonuclease A. Key: —, absorbence at 280 μm ; ----, enzyme activity in arbitrary units.

TABLE 2
MOLECULAR RATIO (RABBIT ANTIBODY/RIBONUCLEASE A)
IN REGION OF ANTIGEN EXCESS: SERUM 619
Rn = Ribonuclease A

Rn N added to 0.16 ml. of serum	Total N in ppt.	Specific N in supernatant	Vol. anti-serum added to supernatant (ml.)	Total N in ppt* from analysis of supernatant	Antibody N precipitated from serum added to supernatant	Per cent Rn N in 2nd ppt.	Rn N in entire supernatant	Rn N in ppt.	Molecular ratio of antibody to antigen in ppt.
32.6	602.7	11.0	0.028	9.0	1.7	2.2	0.4	32.3	1.51
42.4	443.1	180.4	0.12	350.2	229.9	3.1	19.3	26.3	1.36
48.9	340.5	289.5	0.188	594.6	401.5	3.4	30.65	18.3	1.43
55.4	267.2	369.3	0.228	796.6	550.4	3.45	41.2	14.2	1.52
61.9	221.5	421.5	0.252	936.7	655.7	3.5	49.6	12.3	1.45

* All values determined on two thirds of supernatant.

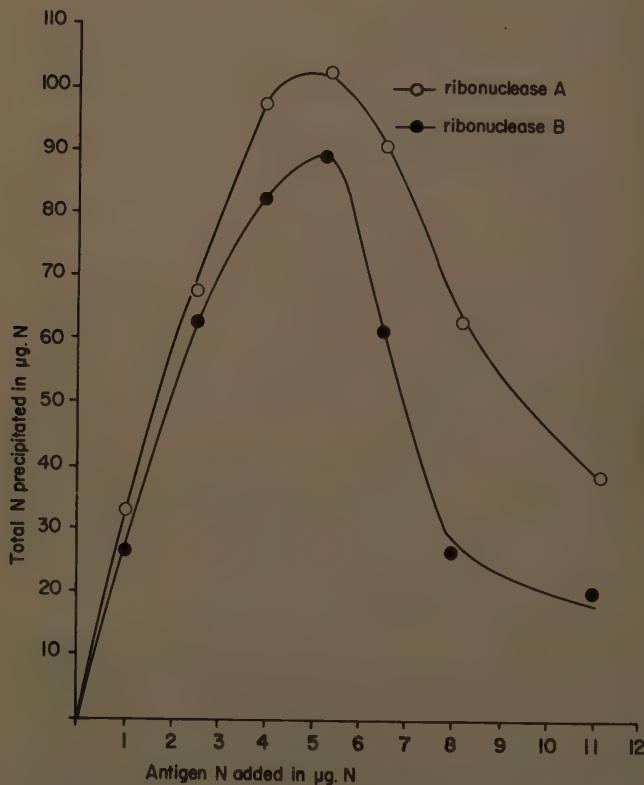


FIGURE 8. Precipitation of total antibody with ribonuclease A and ribonuclease B. Each mixture contained 0.033 ml. gamma globulin (from a pool of ribonuclease antisera (pool G)). Ribonucleases A and B were prepared (Hirs *et al.*^{2b}) from a preparation of crystallized bovine ribonuclease (Armour 381-062); mixtures of antigen and antibody were incubated for 2 hours at 37° C. and were then kept for 6 days at +2° C.

The preliminary removal of part of the antibody, contained in the serum, can also be carried out with ribonuclease B. The residual antibody thus obtained still gives a larger precipitate when mixed with ribonuclease A than when it is mixed with ribonuclease B (FIGURE 9, right hand side). Thus, irrespective of whether the content in antibody is decreased by the preliminary addition of ribonuclease A or B, a residual fraction of antibody is obtained which contains more antibody precipitable by ribonuclease A than precipitable by ribonuclease B.

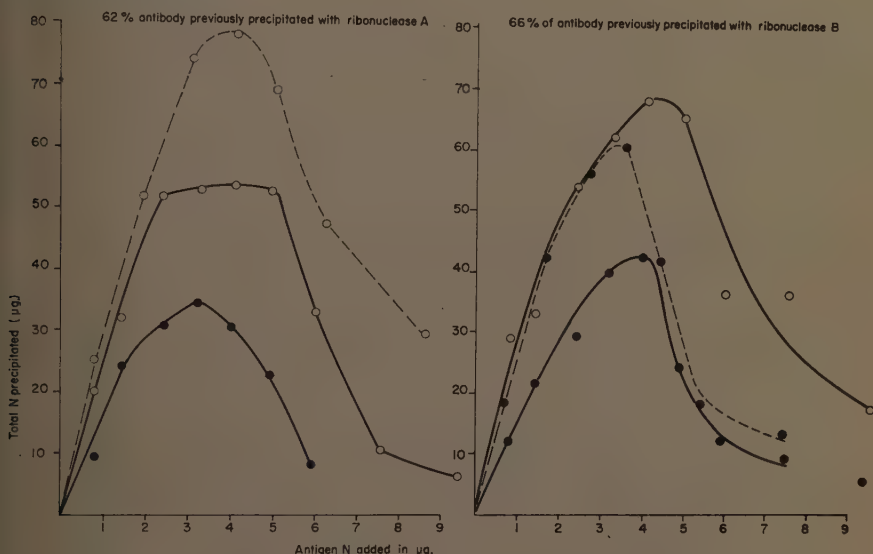


FIGURE 9. The reaction of ribonucleases A and B with antibody from which a fraction was previously removed by precipitation with antigen. Antibody was gamma globulin prepared from a pool of ribonuclease antisera (pool G). Ribonucleases A, B, and gamma globulin employed were the same as in FIGURE 8. In a preliminary step, the ribonuclease antiserum (gamma globulin) was mixed with enough antigen to precipitate 34 to 38 per cent of the total nitrogen precipitable. Constant quantities of the antibody remaining in the supernatant were mixed with increasing quantities of ribonucleases A and B respectively and the precipitated N assayed after standard incubation (incubation as in FIGURE 8). Key: -----, expected quantitative precipitin curve of serum from which a measured amount of antibody has been removed; —, experimentally observed precipitin curve; —○—, quantitative precipitin curve of residual antibody with ribonuclease A; —●—, quantitative precipitin curve of residual antibody with ribonuclease B.

The difference between ribonuclease A and B revealed by their interaction with residual antibody depends on the amount of antibody removed in the preliminary reduction of the antibody content of the ribonuclease antiserum. The greater the fraction of antibody initially removed the greater the difference in the quantity of precipitate formed by mixtures of the residual antibody with ribonuclease A and with ribonuclease B respectively (TABLE 3, last column). This technique could therefore be adapted for the detection of very small quantities of impurity of one form of ribonuclease with the other.

Another interesting feature of the interaction of ribonuclease with residual antibody is the discrepancy between the calculated and the experimentally ob-

served antibody content of residual antibody. The amount of precipitable antibody can be calculated from the direct measurement of the amount of antibody precipitated by antigen in the preliminary reduction of the antibody content of the original serum. This is subject to an error, discussed above, but of no consequence in the following context. A theoretical precipitin curve for the interaction between residual antibody and antigen can be constructed on the assumption that the residual antibody interacts with antigen in exactly the same way as the original (100 per cent) antibody. Such theoretical curves can be compared with the curves experimentally found. It will be seen from TABLE 3 (see also broken lines in FIGURE 9) that the amount of antibody, precipitated by the addition of antigen to residual antibody, is always less than expected. The discrepancy between expected and observed antibody was greater when more of the total antibody had been removed in the preliminary precipitation and was greater when the preliminary precipitation had been

TABLE 3
THE AMOUNT OF NITROGEN PRECIPITATED FROM A MIXTURE OF ANTIGEN
(RIBONUCLEASE A OR B) AND RESIDUAL ANTIBODY

Ribonuclease used in preliminary precipitation	Antibody remaining after preliminary precipitation	Calculated amount of residual antibody		Total N ppt. by Rn B Total N ppt. by Rn A
		Observed amount of residual antibody		
		Antigen added to residual antibody:		
		Rn A	Rn B	
A	100	—	—	0.86
	48	84	(71)	0.74
	38	70	(50)	0.63
B	55	(106)	96	0.74
	34	(89)	70	0.63

carried out with ribonuclease A than when it had been carried out with ribonuclease B.

In addition to ribonucleases A and B the interaction of other fractions with residual antibody was examined. A crude fraction of ribonuclease was separated by chromatography of crystallized ribonuclease on Amberlite CG50 Type II (Hirs *et al.*).^{2a,b} This fraction was subjected to a second chromatographic separation by the same method. A fraction was eluted before ribonuclease B. The interaction of this fraction (fraction C) with residual antibody showed it to precipitate relatively little antibody (FIGURE 10). Whether this was due to the presence of a nitrogenous impurity in fraction C was not further examined.

In other instances it was possible to show that nitrogenous impurities can be separated together with ribonuclease B as isolated by chromatography on carboxymethyl cellulose (FIGURE 11). On examination of the interaction of ribonuclease B (from preparation Sigma R11B-65) it was found that the ribonuclease B so prepared⁵ not only precipitated much less antibody than did ribonuclease A, but that much more antigen was required to precipitate maximum nitrogen with ribonuclease B than was required with ribonuclease A. It was

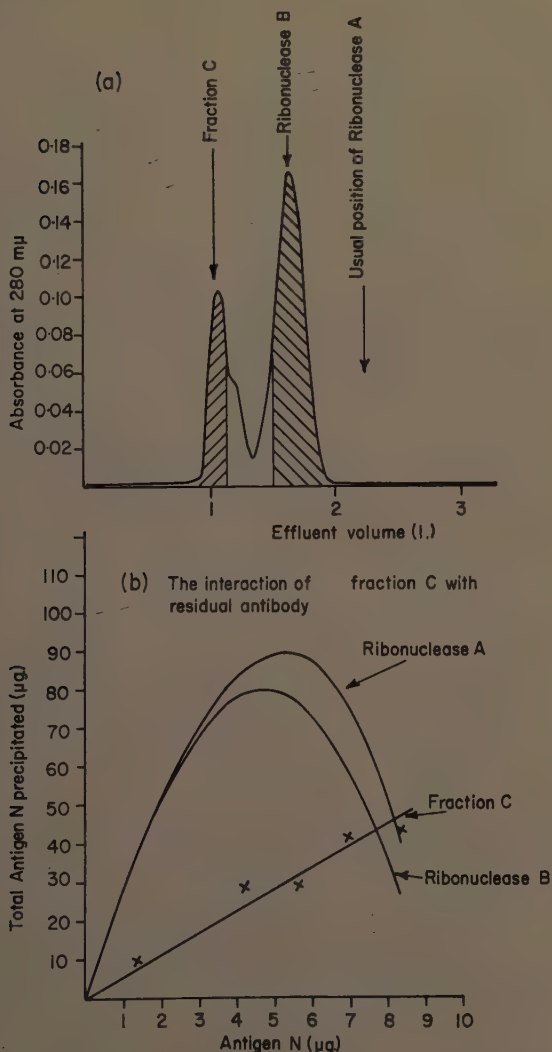


FIGURE 10. Chromatography on Amberlite CG50 Type II of ribonuclease B separated chromatographically from crystallized ribonuclease. (a) Fraction C: crystallized ribonuclease (Sigma R50B-095, R40B-074) was fractionated by the method of Hirs *et al.*^{2b} A crude fraction of ribonuclease B was separated. This fraction was subjected to rechromatography by the same method as shown. Contents of certain tubes (as shown by shaded areas) were pooled and designated as ribonuclease B ($\epsilon_{280}/\epsilon_{260} = 1.52$) and fraction C ($\epsilon_{280}/\epsilon_{260} = 1.37$). (b) The interaction of fraction C, ribonuclease B and ribonuclease A ($\epsilon_{280}/\epsilon_{260} = 1.52$) with residual antibody (gamma globulin, pool B) of which 56 per cent had been previously removed by the addition of ribonuclease A. Antigen and antibody (0.022 ml.) were incubated for 2 hours at 37° C. and then for 6 days at 2° C. Key: —, total amount of N precipitated from residual antibody by ribonuclease A; ----, total amount of N precipitated from residual antibody by ribonuclease B; X—X, total amount of N precipitated from residual antibody by ribonuclease C.

found by electrophoresis in agar and by double diffusion in agar (FIGURE 3) that these preparations of ribonuclease B contained considerable quantities of the "independent" antigen which has been described in the earlier part of this article. Stepwise elution of ribonuclease from columns of carboxymethyl cellulose also failed to yield ribonuclease B free of the "independent" antigen.

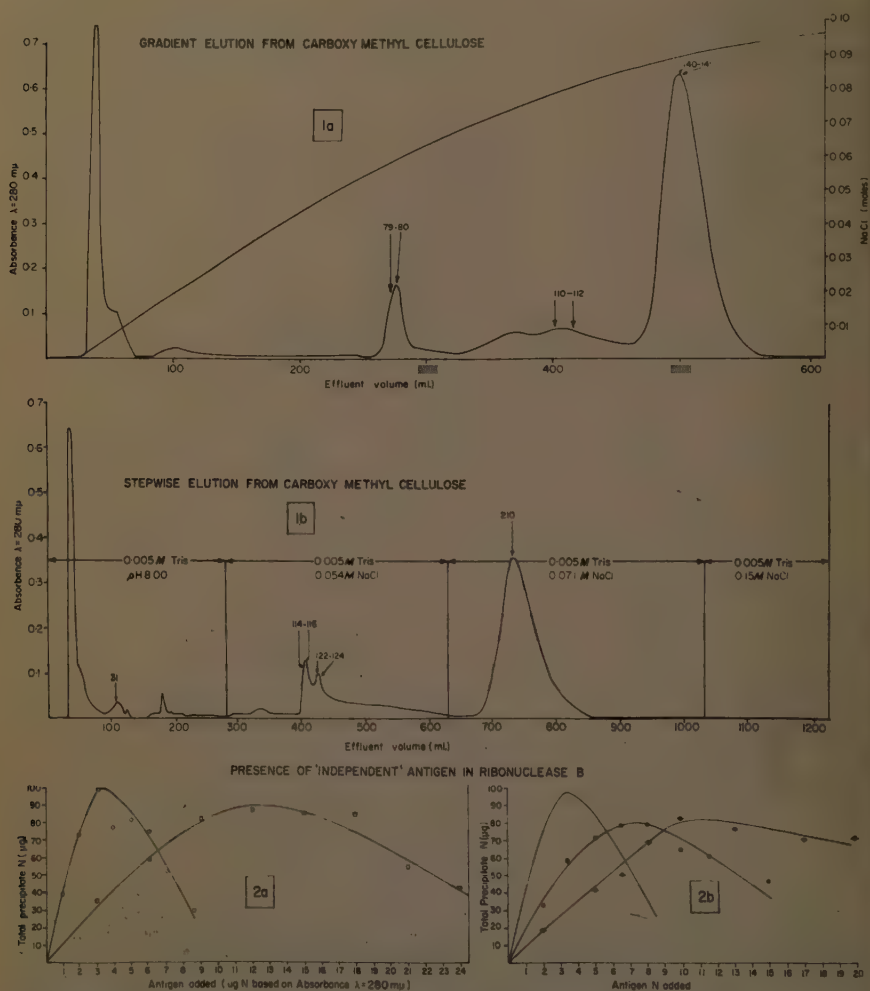


FIGURE 11. The presence of nitrogenous impurities ("independent" antigen) in fractions of ribonuclease B prepared by chromatography on carboxymethyl cellulose. The chromatography on carboxymethyl cellulose of crystallized ribonuclease (Sigma R11B-65) by gradient elution⁵ (1a) and by stepwise elution (1b) and the interaction with residual antibody of fractions obtained by gradient elution (2a) and by stepwise elution (2b). See also FIGURE 3. Residual antibody (pool 6) contained 54 per cent of the antibody originally present. Key:

- Antigen: tube 140-141 (see 1a) $\epsilon_{280}/\epsilon_{260} = 2.08$
- tube 79-80 (see 1a) $\epsilon_{280}/\epsilon_{260} = 1.67$
- tube 114-116 (see 1b) $\epsilon_{280}/\epsilon_{260} = 1.18$
- tube 122-124 (see 1b) $\epsilon_{280}/\epsilon_{260} = 1.26$

It could be demonstrated that the first fraction (FIGURE 11, tubes 114 to 116) of B eluted with 0.054 *M* NaCl, Tris buffer (0.005 *M*, pH 8.0) contained a major portion of the "independent" antigen (FIGURE 11). The complexity of ribonuclease B as revealed by this type of fractionation might be partly due to the presence of a component of the "independent" antigen in this fraction.

The interaction of residual antibody with fractions from denatured ribonuclease (FIGURE 7) was next examined (FIGURE 12). Fractions I and II (FIGURE 7) precipitated slightly more antibody than did ribonuclease A. On the other hand, mixtures of residual antibody and fraction III (FIGURE 7) led to the formation of relatively small quantities of precipitate. Thus fraction III of the denatured crystallized ribonuclease reacted with antibody as if it contained large quantities of antigenically inert protein, whereas the interaction of fraction I and II might be explained by a loss of solubility of the antigen and a consequent relatively low solubility of the corresponding precipitates of antigen and antibody.

It has been established thus far that the antibody N precipitable by ribonucleases A and B can differ by more than 50 per cent if more than 60 per cent of the antibody is removed from the serum before the relative amount of precipitated antibody is measured. The quantitative precipitin test can thus become an extremely sensitive tool in revealing differences between ribonucleases A and B.

The Nature of the Difference Between Ribonucleases A and B Shown by Their Interaction with Residual Antibody

The question next posed must concern the mechanism of interaction underlying the observation presented in the foregoing pages. In view of the reported differences between ribonucleases A and B in only one in the number of free carboxyl groups,⁸ the extent of the immunochemical difference is certainly remarkable. In seeking insight into the reaction, the following possibilities were considered: (1) the different reactivity of the two antigens may be due to the presence of non-specific factors; (2) it may be due to the presence of large quantities of antibodies directed specifically to the particular group (determinant) in which the two enzymes differ; and (3) it may be due to differences in the solubility, rather than in quantity or in specificity, of the antigen-antibody complexes; so that the amount of antibody available for interaction would be the same whether the antigen is ribonucleases A or B. This third possibility implies that the solubility of the compound between residual antibody and ribonuclease B is greater than is the solubility of residual antibody with ribonuclease A.

The importance of nonspecific factors may be considered first. It is conceivable that the presence of complement is of importance in rendering the precipitate insoluble.²⁴ The quantity of complement in an immune serum was therefore reduced by adding 100 μ g. N of a complex of albumin antibody and human albumin to each milliliter of ribonuclease antiserum. The amount of ribonuclease precipitated by a ribonuclease antiserum thus treated was no different from an antiserum not so treated. If after the removal of complement, 50 per cent of the antibody was removed, the amount of ribonuclease

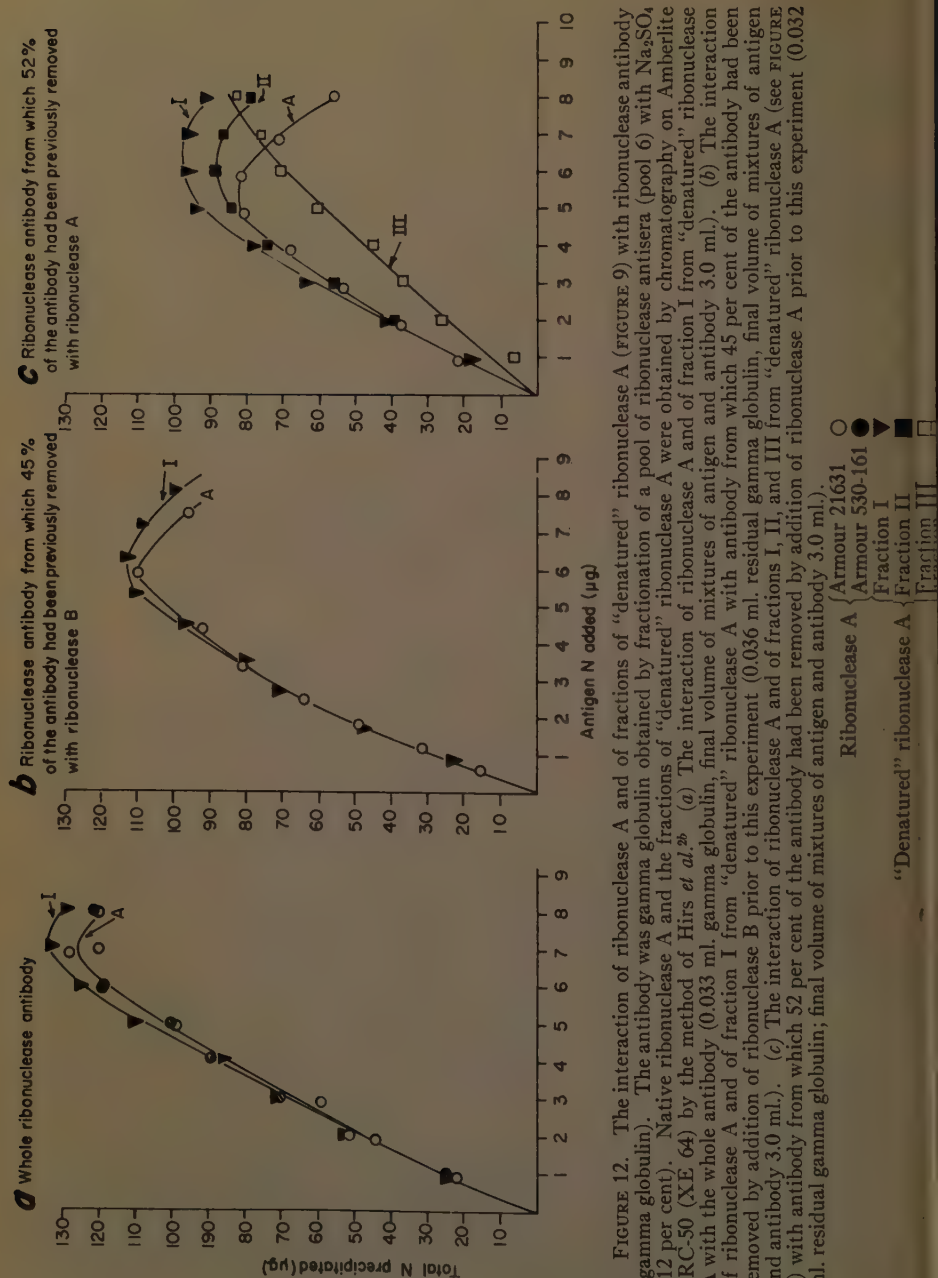


FIGURE 12. The interaction of ribonuclease A and of fractions of "denatured" ribonuclease A (FIGURE 9) with ribonuclease antibody (gamma globulin). The antibody was gamma globulin obtained by fractionation of a pool of ribonuclease antisera (pool 6) with Na_2SO_4 (12 per cent). Native ribonuclease A and the fractions of "denatured" ribonuclease A were obtained by chromatography on Amberlite IRC-50 (XE 64) by the method of Hirs *et al.*²⁶ (a) The interaction of ribonuclease A and of fraction I from "denatured" ribonuclease A with the whole antibody (0.033 ml. gamma globulin, final volume of mixtures of antigen and antibody 3.0 ml.). (b) The interaction of ribonuclease A and of fraction I from "denatured" ribonuclease A with antibody from which 45 per cent of the antibody had been removed by addition of ribonuclease B prior to this experiment (0.036 ml. residual gamma globulin, final volume of mixtures of antigen and antibody 3.0 ml.). (c) The interaction of ribonuclease A and of fractions I, II, and III from "denatured" ribonuclease A (see FIGURE 7) with antibody from which 52 per cent of the antibody had been removed by addition of ribonuclease A prior to this experiment (0.032 ml. residual gamma globulin, final volume of mixtures of antigen and antibody 3.0 ml.).

precipitated was again of the same order of magnitude as it was when the residual antibody was not decomplexed (FIGURE 13). This experiment indicated that complement was not a contributing factor in the phenomenon studied.

Among nonspecific factors the presence of macromolecular impurities must be considered. Such impurities could interact preferentially with one of the two antigens and thus interfere with the reaction of its combinant with antibody. Crystallized ribonuclease contains a substance first eluted in most chromatographic systems of fractionation and characterized by a high absorption at $\lambda = 260 \text{ m}\mu$; it appears to be a contaminant in most purified preparations of ribonuclease B. Was it possible that this substance formed a complex

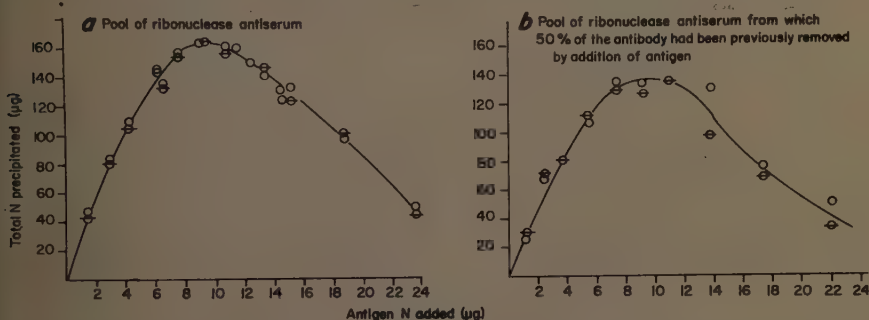


FIGURE 13. The effect of reduction of complement on the interaction between ribonuclease antiserum and crystallized ribonuclease. Decomplementation: a comparison of the interaction of crystallized ribonuclease with undecomplemented serum (pool G) and with serum from which some complement had been removed with a precipitate of an unrelated antigen (human albumin and its antibody). (a) Interaction of ribonuclease with whole immune serum (0.100 ml.) and with immune serum (0.100 ml.) from which complement had been removed. (b) Interaction of ribonuclease with immune serum (0.101 ml.) from which one half the antibody had been previously removed with ribonuclease, and with immune serum (0.102 ml.) from which complement had first been removed, and from which subsequently half the antibody had been removed with ribonuclease. Mixtures of antigen and antibody were kept for 2 hours at 37°C . and then for 6 days at 2°C . Key: —○—, undecomplemented serum; ○—, decomplexed serum.

with ribonuclease B and increased the solubility of complexes between ribonuclease B and antibody? To examine this possibility, a preparation of ribonuclease A which had an exceptionally low ratio ($\epsilon_{280}/\epsilon_{260}$) was selected and its interaction with residual antibody was examined. The difference between this preparation of ribonuclease A and preparations of ribonuclease B was of the same order of magnitude as had previously been found with ribonuclease A, which had the ratio of $\epsilon_{280}/\epsilon_{260} = 1.82$. Furthermore, a comparison was made between two preparations of ribonuclease B that differed in their ratio $\epsilon_{280}/\epsilon_{260}$ but which also differed in that the compound having the lower ratio did not contain any ribonuclease A, whereas the compound with the higher ratio did contain ribonuclease A. The compound having the higher ratio and containing some ribonuclease A precipitated more antibody than the compound having the lower ratio and containing no ribonuclease A. The difference could be explained most adequately by the presence of ribonuclease A in one of the prep-

arations of ribonuclease B in view of the fact, already mentioned, that ribonuclease A, having a very similar ratio $\epsilon_{280}/\epsilon_{260}$ to one of the two preparations of ribonuclease B, precipitated much more antibody than that preparation of ribonuclease B (FIGURE 14).

To confirm the conclusions of this experiment a protein-free fraction, strongly absorbing at $\lambda = 260 \text{ m}\mu$ ($\epsilon_{280}/\epsilon_{260} = 0.76$) was isolated from crystallized ribonuclease by chromatography on carboxymethyl cellulose.⁵ This substance* was then mixed with ribonuclease A and a comparison was made of the interaction with residual antibody of this mixture, of the original ribonu-

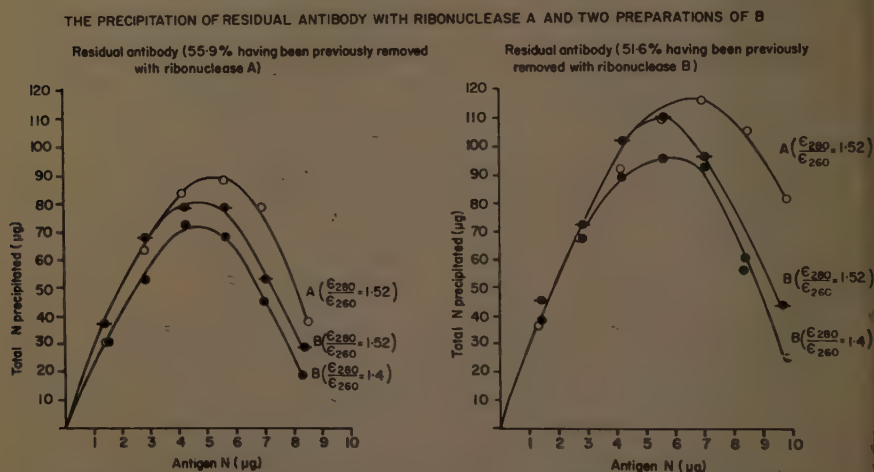


FIGURE 14. A comparison of three fractions of ribonuclease, showing that there is no correlation between ratio of absorbance at $260 \text{ m}\mu$ and $280 \text{ m}\mu$ of the preparations and their interaction with residual antibody. Fractions of ribonuclease obtained by chromatography on Amberlite CG-50 Type II. Mixtures of antigen and residual antibody (0.022 ml. pool B) were kept for 2 hours at 37°C . and subsequently for 6 days at 2°C .

Symbol	Preparation	Source	Content of ribonuclease A	$\epsilon_{280}/\epsilon_{260}$
○	A	(Sigma R50B-095)	100%	1.52
◐	B	(Sigma R50B-095, R40B-074)	Some	1.52
●	B	(Armour 381-062)	None	1.40

lease A and of ribonuclease B. It was again found that ribonuclease A precipitated more N from the residual antibody than would ribonuclease B. Ribonuclease A and ribonuclease A mixed with the protein-free fraction, precipitated the same amount of antibody (FIGURE 15). In immunoelectrophoresis no effect on the mobility or homogeneity of ribonuclease A could be observed as the consequence of the addition of the protein-free fraction. As a consequence of the above experiments the second nonspecific factor could be ruled out from consideration and explanations could be sought in the interaction between antibody and antigen themselves.

* The absorption of this compound was very similar to that of guanine; at acid pH it shows a peak at $257 \text{ m}\mu$ and a shoulder at $272 \text{ m}\mu$; at neutrality there is a peak at $253 \text{ m}\mu$. The ratio of absorptivity between acid and neutral solutions is 1.18.

The fact had to be considered that a moiety of the antibody was capable of combining with only one of the two antigens. This explanation seemed unlikely since residual antibody reacted preferentially with ribonuclease A, irrespective of whether antibody had been initially removed by ribonuclease A or by ribonuclease B (FIGURE 9). This observation excluded the possibility that a proportion of the antibody was adapted to an antigenic site, present on the surface of ribonuclease A but not on the surface of ribonuclease B. From this negative conclusion the problem arose whether the total quantity of anti-

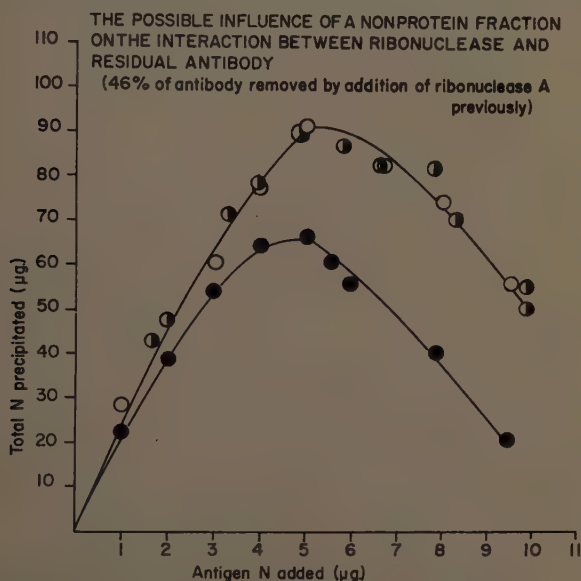


FIGURE 15. A protein-free fraction ($\epsilon_{280}/\epsilon_{260} = 0.76$) was isolated from crystallized ribonuclease (Sigma R110B-092) by chromatography on carboxy methyl cellulose. Ribonuclease A ($\epsilon_{280}/\epsilon_{260} = 1.82$) was isolated from crystallized ribonuclease (Sigma R50B-095) by chromatography on Amberlite CG 50 (Type II), followed by chromatography on carboxy methyl cellulose. Ribonuclease B ($\epsilon_{280}/\epsilon_{260} = 1.40$) was isolated by chromatography on Amberlite CG 50 (Type II) from crystallized ribonuclease. A mixture ($\epsilon_{280}/\epsilon_{260} = 1.32$) was made of ribonuclease A and the protein-free fraction. The various antigens were mixed with residual antibody (0.027 ml., pool 6), incubated for 2 hours at 37° C. and then for two days at +2° C. Key: —○—, ribonuclease A; —●—, ribonuclease A mixed with protein-free fraction; —●—, ribonuclease B.

body available for combination with ribonuclease A and with B was really different. By quantitative precipitin tests a considerable difference had been demonstrated, but was this difference a reliable indication of the reactivity of the total antibody in the system? This question was examined by estimating the amount of antibody in terms of its inhibitory capacity.²¹ By these means it could be conclusively demonstrated that ribonuclease antisera contained a considerable proportion of antibody which, although combining with the enzyme, would not precipitate with it. The relative amount of antibody contained in the original serum and in residual ribonuclease antiserum was determined by quantitative precipitin test and by measurement of inhibitory capacity. The latter test was carried out by determining neutralization curves

based on the enzyme activity of mixtures of antigen and antibody. Constant quantities of enzyme were mixed with different quantities of antibody; after 30 min. incubation at 37° C. the mixtures were added to the substrate, ribose nucleic acid. The enzyme activity was then determined manometrically. Enzyme activity of mixtures of ribonuclease A and antibody were expressed

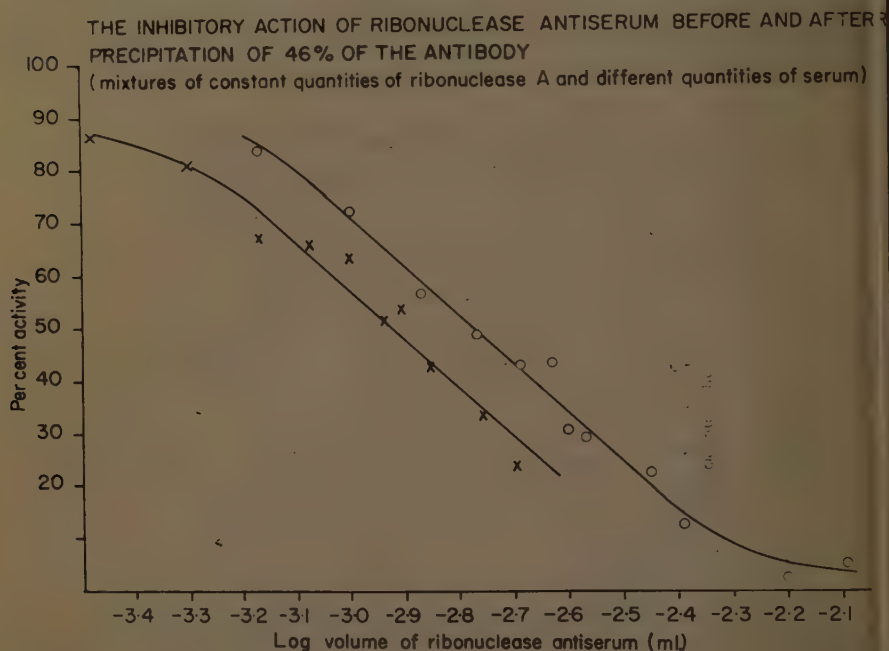


FIGURE 16. A comparison of antibody content before and after removal of some of the antibody by specific combination with ribonuclease A. Enzyme activity was measured manometrically by determining the CO_2 evolved from a bicarbonate buffer as a consequence of the liberation of phosphate groups in nucleic acid. Activity of antibody was estimated by the inhibition of enzyme action. Different amounts of antibody (serum, pool 6) were added to constant amounts of enzyme ($0.5 \mu\text{g}$. N). The activity of each mixture was expressed as the percentage of the activity of enzyme in the absence of antibody. The percentage activity was plotted as a function of the logarithm of the volume of ribonuclease antiserum in each mixture. The displacement between the linear portions of the resulting curves is the log of their relative content in antibody. By this method the residual antibody was shown to contain 70 per cent of the antibody in the original serum from which no antibody had been removed. Measurement by quantitative precipitin assay had given a value of 54 per cent. Key: —X—, ribonuclease antiserum containing 100 per cent antibody; —O—, ribonuclease antiserum from which 54 per cent of the total antibody, judged by precipitin assay, had been previously removed by precipitation with ribonuclease A.

in terms of the activity of the enzyme in the absence of antibody. The percentage activity thus obtained was plotted as a function of the logarithm of the volume of ribonuclease antiserum in each mixture. Neutralization curves were measured with mixtures containing the original serum and the residual serum respectively. The horizontal displacement between the linear portions of these two neutralization curves is the logarithm of the ratio of the antibody content of the two sera (FIGURE 16). The antibody content of the residual

serum was 70 per cent (as a fraction: 0.703 ± 0.036) of the antibody of the original serum, judged by inhibitory capacity. By quantitative precipitin assay the residual serum was estimated to contain 54 per cent of the antibody present in the original serum. Thus approximately 16 per cent of the antibody present in the original serum could combine with antigen but could not precipitate with it.

Neutralization curves were next constructed from the measured activity of mixtures containing residual serum and ribonuclease A and B respectively.

A COMPARISON OF THE INHIBITION OF RIBONUCLEASES A AND B BY RIBONUCLEASE ANTISERUM FROM WHICH 46% OF THE ANTIBODY HAD BEEN PREVIOUSLY REMOVED BY THE ADDITION OF ANTIGEN

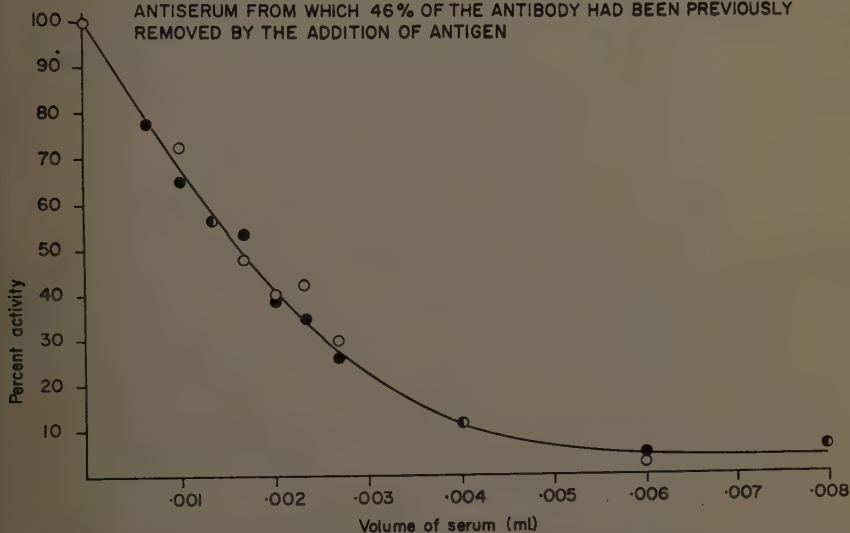


FIGURE 17. The effect of residual antibody on the catalytic action of ribonuclease A and B. Prior to the experiment some antibody was removed by the addition of ribonuclease A to the serum (46 per cent as judged by quantitative precipitin assay, 30 per cent as judged by inhibitory capacity).

Mixtures of enzyme (constant quantity, 0.5 μ g. N ribonuclease A or 0.49 μ g. N ribonuclease B) of residual antibody (from serum, pool 6) (varying quantities) were incubated at 37° C. for 30 min. and then were added to ribose nucleic acid. The activity of the mixtures of antigen-antibody was measured manometrically (CO_2 evolved 10th to 35th min.) and expressed as a percentage of the activity of the enzyme in the absence of antibody. Key: —○—, ribonuclease A; —●—, ribonuclease B.

In FIGURE 17, the percentage activity is plotted as a function of the volume of serum. As will be seen from the figure, ribonucleases A and B are inhibited to the same extent. It follows, therefore, that the amount of antibody combining with ribonucleases A and B is the same. The difference manifested in the interaction of the same reactants in the quantitative precipitin test must be due to the relative solubility of the complex of ribonuclease A with residual antibody and of ribonuclease B with residual antibody.

It is unlikely that this difference between combination and precipitation can be attributed to the dissociation between the antigen and the antibody. If such a difference existed in interaction of ribonuclease A with residual antibody and of ribonuclease B with residual antibody, this would manifest itself in the

neutralization curves by a difference in the slope of the neutralization curves of the two enzymes. Such a difference, however, has not been observed (FIGURE 17). It therefore seems most probable that the differences between ribonucleases A and B observed in the quantitative precipitin tests are due to the fact that the differences in surface between ribonucleases A and B lead to the formation of complexes between antigen and antibody which would have a different solubility; as a consequence, a proportion of the antibody molecules would be soluble with both antigens, and another proportion would be insoluble with both antigens. A third fraction of antibody, with intermediate properties, would be soluble or insoluble, depending on the contribution of the antigen to the solubility of the complex. It may thus be concluded that the immunochemical differences between ribonucleases A and B, manifested so decisively with residual antibody, can probably find their explanation in the relative contribution to the solubility of the two antigens when combined with antibody.

Discussion

Crystallized ribonuclease contains 5 forms of the enzyme that appear to differ from one another by their electrophoretic mobility. With 3 of the 5 forms described, the molecules, identified by immunoelectrophoresis, can be identified as chromatographic entities. Taking the nomenclature of Hirs *et al.*^{2a,b} as a starting point and the classification of serum proteins as a model, the multiple forms of ribonuclease may be designated as A₁ (FIGURE 4), A₂ (FIGURE 5), B₁, B₂ (FIGURES 3, 4), and pre-A (FIGURE 6); A₁ and B₂ are identical with the principal components described by Martin and Porter and by Hirs *et al.*^{2a,b} B₁ might be identical with des-lysino ribonuclease.^{22a,b,23}

The possibility can be ruled out that some of the minor components (A₂, B₁, pre-A) may be complexed with the protein-free material ($\epsilon_{280}/\epsilon_{260} = 0.76$) that is known to be present in crystallized ribonuclease. This possibility has been excluded in the case of ribonuclease A₁ and ribonuclease B₂ and is very unlikely to apply in the case of the other forms of the enzyme. In fact, experiments in which ribonuclease A₁ was mixed with the protein-free fractions ($\epsilon_{280}/\epsilon_{260} = 0.76$) and in which the resulting mixture was subjected to immunoelectrophoresis, showed that the mobility of ribonuclease A is independent of the presence of the protein-free fraction.

By means of a method of partial absorption, a very considerable difference was demonstrated between ribonucleases A₁ and B₂. Factors responsible for these differences have been analyzed experimentally and were shown to be probably attributable to the contribution of antigen to the solubility of the compound of antigen and antibody. In the light of this interpretation, the observations made with residual sera containing different percentages of residual antibody can be readily understood. In these experiments (TABLE 3) a discrepancy was noted between the calculated and the experimentally observed antibody content of residual antibody and furthermore the difference between the amount of antibody precipitated by ribonuclease A and ribonuclease B was greater the smaller the fraction of remaining antibody. Both these observations are attributable to the role of the soluble complex of antigen and antibody.

The original serum contained precipitable as well as nonprecipitable antibody. The proportion between precipitable and nonprecipitable antibody was changed after removal of some precipitable antibody. Consequently the residual antibody contained much more nonprecipitable antibody than did the original serum. Thus an antibody originally heterogeneous, with respect to the solubility of antibody-antigen complex, apparently becomes progressively enriched in nonprecipitable antibody with removal of portions of precipitating antibody. As the proportion of soluble antibody in the serum increases so does the proportion of $\frac{\text{antibody precipitable by ribonuclease } A_1}{\text{antibody precipitable by ribonuclease } B_2}$.

In the chromatographic fractionation of denatured ribonuclease A a fraction was isolated that precipitated more antibody than did any native fraction of ribonuclease. This phenomenon was again attributable to the presence of a fraction of antibody combining with antigen to give a relatively soluble complex with antigen and to changes in the denatured antigen leading to a relatively insoluble complex of antigen and antibody (FIGURE 12).

In the experiments described, the residual antibody revealed differences between related antigens in the quantitative precipitin test, but not in tests based on enzyme neutralization (FIGURE 17). This is not surprising, since highly "avid" sera were employed throughout that would combine so firmly with antigen that small differences in reactivity with antigen would be difficult to detect. Such differences have, however, been found in other systems.

Pollock has applied the method of residual antibody imaginatively in his characterization of the multiple forms of penicillinase.¹⁶ He has relied entirely on tests of the enzyme activity of mixtures of penicillinases and antibody and has found marked differences in the penicillinases of strains of *Bacillus cereus* 569 and 5/B.¹⁷ Partial absorption of antibody with either 5/B or 569 penicillinase left a residual antibody that had a much higher inhibitory capacity with respect to 569 penicillinase activity than it had with respect to 5/B penicillinase activity. Pollock suggested that antibodies of low avidity ("inefficient" antibodies) are left behind selectively on partial absorption. These "inefficient" antibodies would nevertheless be relatively more efficient against the homologous antigen.

We have described the effect of the solubility of the antigen-antibody complex on the interaction of two very similar antigens with residual antibody. From the interaction of residual antibodies with penicillinase it may be concluded that "avidity" of antibody may be a factor, under other circumstances, which may play a part in this type of interaction.

It may well be that the differences found in neutralization tests with antibody and the two penicillinases, on one hand, and with antibody and the two ribonucleases, on the other, are not attributable to the respective structural differences between the members of each of the two pairs of antigens, but to differences in the over-all avidity of the sera used in the investigation of ribonuclease and of penicillinase. The sera used in the investigation of ribonuclease were uniformly of very high avidity. This was a deliberate choice, since it made an analysis of the interaction of ribonuclease A_1 and ribonuclease B_2 much simpler than it would otherwise have been. However, it is clear that in applications of this method to other closely related antigens, the use of both

avid and of nonavid sera could be of considerable advantage. The use of sera of great avidity probably confines the application of the discriminating potential of the residual antibody to the quantitative precipitin assay. This assay, however, is only applicable with preparations which are essentially freed of unrelated antigens, whereas a test that would be based on discrimination by changes in activity due to inhibition of enzyme by antibody^{19a,b} would be applicable to relatively impure preparations of antigens.

The general applicability of the quantitative precipitin test with residual antibodies still remains to be explored. It has been shown in the foregoing pages that this test was useful in the examination of denatured fractions of ribonuclease as well as of ribonucleases A₁ and B₂. In another antigen-antibody system, bovine hemoglobin A and B,¹⁸ in which the antigens differed to about the same extent as the two ribonucleases, a difference between the two antigens could not be established by the interaction with residual antibody (Bangham and Cinader, unpublished).

Summary

Five forms of ribonuclease could be found in crystallized bovine pancreatic ribonuclease. Ribonucleases A and B were isolated and considerable differences between these two antigens were found in their interaction with antibody, from which a fraction of antibody had been previously removed with ribonuclease A or B. This method was also applied to the study of fractions of denatured ribonuclease and immunochemical differences between them and native ribonuclease were found. The mechanism underlying the difference of the activity was investigated. It was shown to be attributable to the different solubility of antigen and antibody complexes formed between residual antibody and ribonuclease A and B, respectively.

References

1. MARTIN, A. J. P. & R. R. PORTER. 1951. Chromatographic fractionation of ribonuclease. *Biochem. J.* **49**: 215.
- 2a. HIRS, C. H. W., W. H. STEIN & S. MOORE. 1951. Chromatography of proteins. Ribonuclease. *J. Am. Chem. Soc.* **73**: 1893.
- 2b. HIRS, C. H. W., S. MOORE & W. H. STEIN. 1953. A chromatographic investigation of pancreatic ribonuclease. *J. Biol. Chem.* **200**: 493.
- 3a. ALBERTY, R. A., E. A. ANDERSON & J. W. WILLIAMS. 1948. Homogeneity and the electrophoretic behaviour of some proteins. *J. Phys. & Colloid Chem.* **52**: 217.
- 3b. ANDERSON, E. A. & R. A. ALBERTY. 1948. Homogeneity and the electrophoretic behaviour of some proteins. II. Reversible spreading and steady-state boundary criteria. *Ibid*: 1345.
4. RAACKE, I. D. & C. H. LI. 1954. Electrophoretic inhomogeneity of crystalline ribonuclease. *Biochim. Biophys. Acta.* **14**: 290.
5. TABORSKY, G. 1959. Chromatography of ribonuclease on carboxymethyl cellulose columns. *J. Biol. Chem.* **234**: 2652.
6. ÅQVIST, S. E. G. & C. B. ANFINSEN. 1959. The isolation and characterization of ribonucleases from sheep pancreas. *J. Biol. Chem.* **234**: 1112.
7. McDONALD, M. R. 1948. A method for the preparation of protease-free crystalline ribonuclease. *J. Gen. Physiol.* **32**: 39.
8. TANFORD, C. & J. D. HAUENSTEIN. 1956. Identification of the chemical difference between chromatographic components of ribonuclease. *Biochim. Biophys. Acta.* **19**: 535.
9. HAKIM, A. A. 1956. Ribonuclease specific activity. A new solvent system for the isolation of the polynucleotides on filter paper. *Enzymologia.* **17**: 315.

10. SHAPIRA, R. & S. PARKER. 1960. Artificially induced microheterogeneity in ribonuclease. *Biochem. Biophys. Research Comm.* **3**: 200.
11. CINADER, B. & J. H. PEARCE. 1956. Immunochemical studies on bovine ribonuclease. *Brit. J. Exptl. Pathol.* **37**: 541.
12. CINADER, B. & J. H. PEARCE. 1958. Immunological approaches to the study of ribonuclease. : 240. *In* Symposium on Protein Structure. A. Neuberger, Ed. Methuen. London, England.
13. GRABAR, P. & C. A. WILLIAMS. 1953. Méthode permettant l'étude conjuguée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines. Application au sérum sanguin. *Biochim. et Biophys. Acta.* **10**: 193.
14. CINADER, B. & J. M. DUBERT. 1955. Acquired immune tolerance to human albumin and response to subsequent injections of diazo human albumin. *Brit. J. Exptl. Pathol.* **36**: 515.
15. CINADER, B. & J. H. PEARCE. 1958. The specificity of acquired immunological tolerance to azo proteins. *Brit. J. Exptl. Pathol.* **39**: 8.
16. POLLOCK, M. R. 1956. An immunological study of the constitutive and the penicillin-induced penicillinases of *Bacillus cereus*, based on specific enzyme neutralization by antibody. *J. Gen. Microbiol.* **14**: 90.
17. POLLOCK, M. R. 1956. The cell-bound penicillinase of *Bacillus cereus*. *J. Gen. Microbiol.* **15**: 154.
18. BANGHAM, A. D. 1957. Distribution of electrophoretically different haemoglobins among cattle breeds of Great Britain. *Nature.* **179**: 467.
- 19a. CINADER, B. 1953. Antigen-antibody interaction using enzymes as antigens. *Biochem. Soc. Symposium.* **10**: 16.
- 19b. CINADER, B. 1957. Antibodies against enzymes. *Ann. Rev. Microbiol.* **11**: 371.
20. HIRS, C. H. W., S. MOORE & W. H. STEIN. 1956. Peptides obtained by tryptic hydrolysis of performic acid-oxidized ribonuclease. *J. Biol. Chem.* **219**: 623.
21. BRANSTER, M. V. & B. CINADER. 1961. Interaction between bovine ribonuclease and antibody; a study of the mechanism of enzyme inhibition by antibody. *J. Immunol.* **87**: 18.
- 22a. EAKER, D. & T. P. KING. Unpublished results. *Quoted in*: CRAIG, L. C., T. P. KING & W. KONIGSBERG. 1960. Homogeneity studies with insulin and related substances. *Ann. N. Y. Acad. Sci.* **88**(2): 571.
- 22b. KING, T. P., D. A. YPHANTIS & L. C. CRAIG. 1960. Distribution studies with bovine plasma albumin. *J. Am. Chem. Soc.* **82**: 3350.
23. BROWN, R. K. 1960. The function of the N-terminal lysine in ribonuclease immune reactivity. *Federation Proc.* **19**: 201.
24. WEIGLE, W. O. & P. H. MAURER. 1957. The effect of complements on soluble antigen-antibody complexes. *J. Immunol.* **79**: 211.

Discussion on Nomenclature

A. SAMUELS (*Department of Pathology, Dartmouth Medical School, Hanover, N.H.*): If the structures to which we are thinking of applying the name isozymes are due to changes in primary structure of the enzymes based upon the selection of particular proliferating cells in an organ that therefore result in a change in the proportion in certain cell types during disease or physiological stress it might therefore be a reproducible phenomenon. However, if there are conformation changes that occur (nonnucleic acid mediated changes) that result in metastable states that may be changed back into the original form as the conditions vary slightly we might not be justified in calling these isozymes. Examples of this type of change is the penicillinase of Pollock and Citri mentioned by Schlamowitz and the case of myosin A (a and b forms) that are interconvertible as found recently by Brahms.* The changes in disease states mentioned by Allen and in the case of denervation atrophy on which I have reported† may be either of the above types of change. The third type of change that may occur may be the conformation changes that take place during substrate com-

* *J. Am. Chem. Soc.* 1959. **81**: 4997.

† *The Physiologist*. 1957. **1**: 24.

ination,* for which there is now immunoenzymological evidence and optical rotation evidence.† This last type of change might also conceivably be called an isozyme, although it probably would be detected by the current "equilibrium" techniques of starch electrophoresis and chromatography only if the enzyme substrate complex were very firm.

* The Enzymes, Lumry, R, Chap. 4; Koshland, D. Chap. 7, Schellman, J., and Lang, L. Chap. 10. Editors, Boyer, Lardy and Myrbäck.

† SAMUELS, A. 1961. Biophys. J. 1(6): 437; Biophys. Soc. 1961. 5 TB-9.

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